Detection of Shifts in Microbial Functional Gene (*nosZ* and *nifH*) Distribution Due to Long Term Warming of a High Arctic Soil

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ABSTRACT

Climate change is expected to increase annual arctic temperatures by up to 5 °C and winter arctic temperatures as much as 7 °C within the next century. High Arctic coastal lowlands, such as at Alexandra Fiord, Ellesmere Island, have greater productivity and species diversity than the surrounding desert-like plateaus. Warming due to polyethylene open-topped chambers (OTCs) has been shown to increase plant productivity and cause community shifts compared to adjacent control plots. Changes in the soil environment have also been noted and will affect microbial activity and community composition. Long term elevated temperatures may result in changes to the community structure of these organisms. By studying terminal restriction fragment length polymorphisms (T-RFLPs) of genes associated with nitrogen cycling, we can examine variations in the nitrogen cycling microbial community.

The objective of this study was to detect shifts in denitrifying and nitrogen fixing soil microbial communities by measuring changes in functional gene frequency, abundance and/or genotypic richness of *nos*Z and *nif*H respectively. The study area encompassed five high arctic sites that differed by dominant plant community, soil parent material and/or moisture regime and that had been subjected to a thirteen year warming experiment. Four OTCs plus four adjacent control plots were sampled at each of these locations in order to investigate differences in these gene communities due to site, depth, and treatment. Samples were recovered from the top and bottom 5 cm of soil cores; these ranged from 13 cm to 44 cm deep. DNA extractions from soil samples were

tested with two different primer pairs. Functional genes targeted included those that code for nitrous oxide reductase (*nos*) and nitrogenase (*nif*). Differences in frequency and relative abundance of terminal restriction fragments (TRFs) were assessed graphically by Non-metric Multidimensional Scaling (NMS) and tested statistically with permutational multivariate ANOVA (PERMANOVA). Genotypic richness was examined by testing the difference in number of TRFs with nested and one-way ANOVA.

Functional gene frequency and relative abundance was shown to differ overall by site in NMS ordinations of both denitrifying and nitrogen fixing communities. PERMANOVA tests also suggested a significant difference in the relative abundance of denitrifying TRFs, and both the frequency and relative abundance of nitrogen-fixing TRFs by depth over all sites. Differences in genotype richness were detected between sites, at different depths, and due to treatment for both communities. In general, denitrifying and nitrogen fixing community richness decreased with soil depth and with OTC treatment.

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LIST OF ACRONYMS

SM	Sedge Meadow
CH	Cassiope Heath
RW	Riverside Willow
UG	Upland Granite
UD	Upland Dolomite
nir	nitrite reductase
nos	nitrous oxide reductase
nif	dinitrogenase reductase
amo	ammonium monooxygenase
OTCs	open-topped chambers
T-RFLPs	terminal restriction fragment length polymorphisms
TRFs	terminal restriction fragments
PCR	polymerase chain reaction
RT-PCR	real time PCR
DGGE	denaturing gradient gel electrophoresis
DEA	denitrification enzyme assay
ARA	acetylene reduction assay
GC	gas chromatography
CEC	cation exchange capacity
OM	organic matter
SWC	soil water content
NMS	Non-metric Multidimensional Scaling
PERMANOVA	permutational multivariate ANOVA
ITEX	International Tundra Experiment
IPCC	Intergovernmental Panel on Climate Change
ACIA	Arctic Climate Impact Assessment

Chapter 1. Literature Review

1.1 Climate change and arctic systems

Climate change is expected to disproportionately affect arctic and antarctic latitudes (Hassol 2004, Maxwell *et al.* 1992). Positive feedbacks due to changes to polar weather patterns and ecosystems will further affect global climate. Current climate change models assume a doubling of atmospheric carbon dioxide (CO_2) concentration in less than fifty years. This prediction is based upon rates of CO_2 increase measured recently; the concentration rose steadily after the last ice age, but has risen dramatically from the 19^{th} century to present day. It is the accumulation of radiative gases such as CO_2 in the atmosphere that reinforces the greenhouse effect. Radiative gases have the capacity to absorb outgoing thermal energy and re-radiate it back toward the earth's surface. This blanket of gases is what makes our planet habitable, but it is at the root of excess global warming. The effects are dramatic: within 100 years, annual arctic temperature may be 3-5 °C higher, with winter temperatures as much as 4-7 °C higher over land than they are currently (Hassol 2004, Maxwell *et al.* 1992).

There are other gases implicated in climate change, and although they are present in much smaller concentrations, their thermal capacity on a per molecule basis, and their persistence in the atmosphere is far greater (Maxwell *et al.* 1992). Nitrous oxide (N_2O), a product of nitrogen transformation cycles, such as denitrification and nitrification, is one of these.

Though many climate change models may agree that certain variables are crucial inputs, the outcomes predicted are not always the same and in fact are often contradictory (Maxwell *et al.* 1992). The exact changes, their feedbacks, and their implications are highly debatable. Most researchers agree that overall annual global precipitation will increase, and, like temperature, will be greater at the poles. Higher winter temperatures and rainfall in the arctic will decrease residual spring snow cover and promote permafrost thawing (Hassol 2004). Contradictions arise regarding net soil moisture; some suggest an increase due to melting of the permafrost layer and earlier snowmelt, while others hint at a decrease due to better drainage and increased evaporative losses (Hassol 2004, Kane *et al.* 1992, Maxwell *et al.* 1992). A combination of these two predictions may be possible with wet, coastal areas getting wetter, and dry, inland areas getting drier (Maxwell *et al.* 1992).

Increased surface temperature resulting in heat transfer to the soil via conduction has also been assumed (Kane *et al.* 1992). The greatest warming is predicted to occur in the winter, but the soil at -15 cm should still remain approximately 3°C warmer than usual throughout the summer (Kane *et al.* 1992). Recent studies have shown that air temperature can be raised to predicted levels by experimental warming with greenhouses without a proportional increase in soil temperature (Jonasson *et al.* 1993, Robinson 2002). This challenges the assumption of a deeper active layer and more nutrient cycling with warming.

1.2 Arctic research sites

Some coastal lowlands in the high arctic have greater productivity and species diversity due to different temperature and moisture regimes than the surrounding desert-like plateaus (Freedman *et al.* 1994). These unique ecosystems provide excellent study sites for the predicted changes due to global warming because of seasonal extremes in solar energy and precipitation, and because these areas are expected to experience the greatest transitions (Freedman *et al.* 1994, Maxwell *et al.* 1992). Alexandra Fiord is a High Arctic research site established by the International Tundra Experiment (ITEX) (Figure 1). See Arft *et al.* (1999), Rustad *et al.* (2001) and Walker *et al.* (2006) for comprehensive summaries of climate change experiments at this and other subarctic and alpine sites.

Polyethylene open-topped chambers (OTCs) have been installed across various gradients at the Alexandra Fiord site, and many studies have taken place over the last decade. Warming due to these OTCs, which generate a small greenhouse effect, has been shown to increase plant productivity and create community shifts compared to adjacent control plots on wet, moist, and dry lowland, and on granitic and dolomitic upland sites (Freedman *et al.* 1994, Rolph 2003).

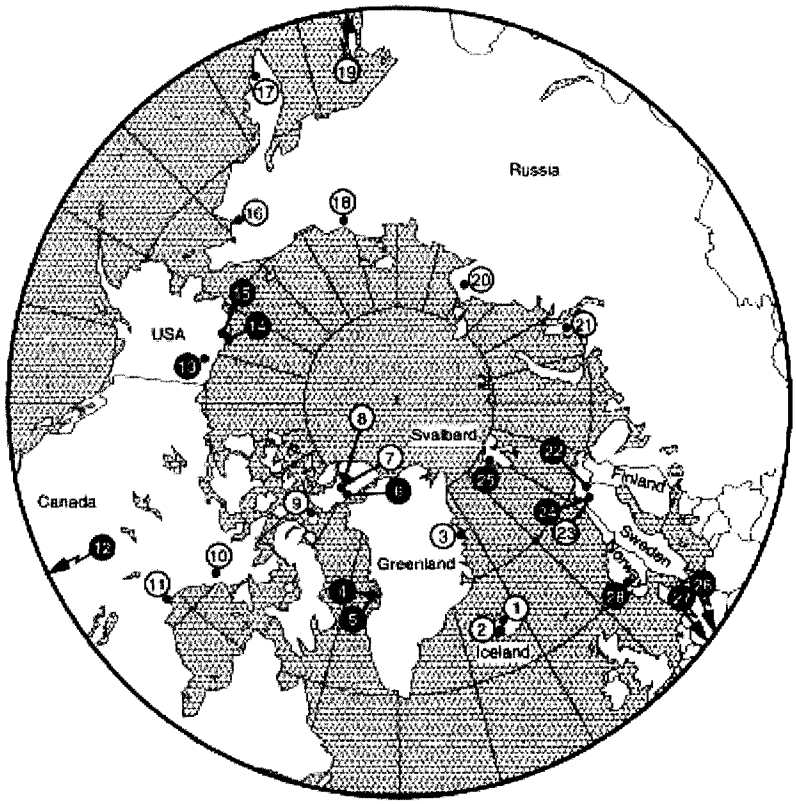

Figure 1: The High Arctic site at Alexandra Fiord (6) is one of a number of arctic, subarctic and alpine ITEX study sites (modified from Arft *et al.* 1999).

1.3 Soil N cycling and plants

The soils of a polar oasis can differ dramatically from those of the surrounding polar desert (Muc *et al.* 1994). Even slight increases in both temperature and precipitation can lead to deeper active layers, higher rates of chemical transformations and ultimately, more nutrient availability and plant productivity. The primarily granitic-derived soils of the Alexandra Fiord lowland, although less developed than those of temperate regions, have more organic matter, higher soil moisture levels and increased cation exchange capacity (CEC) than upland

samples. Despite this, all lowland sites and both the granitic and dolomitic upland sites are still considered nutrient (especially nitrogen) limited (Muc *et al.* 1994).

Nitrogen gas (N_2) comprises 78% of our atmosphere, and its fixation by microbes is the major source of new nitrogen (N) in soils (Paul and Clark 1996). Other inputs of N such as fertilization and atmospheric deposition are not relevant in remote arctic regions (Paul and Clark 1996). It is possible that future arctic environments will be less nitrogen limited due to a predicted increase in nitrogen fixation (Chapin and Bledsoe 1992). The potential increase is attributed to favourable enzymatic activity at higher temperatures, and to elevated levels of carbon dioxide and moisture that will benefit the aquatic and photosynthetic nitrogen fixers (diazotrophs) (Chapin and Bledsoe 1992).

Nutrient cycling in this arctic environment could be enhanced with the additional warming predicted by climate change models (Berendse and Jonasson 1992). This increase in soil fertility and nitrogen availability will affect plant-soil dynamics and predominant soil nutrient cycles such as net mineralization versus net immobilization (Berendse and Jonasson 1992). Mineralization is simply the degradation of organic forms of elements such as nitrogen to their mineral forms; ammonium (NH_4^+) is then immobilized for microbial growth as required, and the excess may accumulate in the soil (Paul and Clark 1996). Arctic plants are already adapted to low temperatures, so the predicted increased productivity will be limited by nutrient availability which is in turn mediated by soil response to

warming (Chapin III *et al.* 1992). It is likely that changes in soil characteristics, especially moisture, will drive the changes in other biogeochemical cycles (Chapin III *et al.* 1992).

In general, net soil mineralization is low or even negative during the arctic growing season with any excess nutrients sequestered in microbial biomass (Jonasson *et al.* 1999, Nadelhoffer *et al.* 1992). Microbes compete strongly for nutrients throughout the growing season, but as their populations decline in the winter, it is possible that nutrients are released and available to plants (Jonasson *et al.* 1999). These plants are adapted to low nutrient levels and therefore any significant changes in availability can lead to changes in plant productivity and species composition. Although increased net N mineralization with warming has been predicted (Nadelhoffer *et al.* 1992), recent studies have not confirmed that it will be affected by short term increases in soil temperature of a few degrees Celsius (Jonasson *et al.* 1999, Schmidt *et al.* 1999). The variability in net mineralization between soils from different plant communities is often more than any differences observed due to temperature treatments (Schmidt *et al.* 1999).

Although in past studies increases in soil temperatures of 1-1.5°C at 5-7 cm deep were accomplished with OTCs, greater net N mineralization was only observed at a one high altitude site in the winter; no changes in microbial immobilization were noted for any treatments. The techniques used in this study suggest that microbes are very strong competitors for soil nutrients in all seasons, and that an

increase in mineralization with their population decline at the end of a growing season cannot be considered a general rule (Schmidt *et al.* 1999). Longer term warming experiments have revealed increases in microbial immobilization due to changes in soil properties that are attributed to sustained elevated temperatures (Rolph 2003). Shorter term changes in N cycling and availability have modified the plant community composition over time and the feedbacks are now notable as changes in litter quality and plant productivity (Rolph 2003).

1.4 Soil N cycling and microbes

Changes in soil moisture status (associated with global warming) will impact microbial processes such as decomposition in all arctic ecosystems; the resulting combination of moisture and temperature will dictate organic matter (OM) turnover rates, and ultimately NH_4^+ availability via mineralization (Paul and Clark 1996, Nadelhoffer *et al.* 1992). The predicted and measured changes in the soil environment with increased temperature are not limited to nutrient mineralization and subsequent availability to plants; microbial activity and community composition are affected (Nadelhoffer *et al.* 1992).

Depending upon moisture, nutrient availability, and timing, the process of nitrogen fixation is shared across a range of soil organisms (Paul and Clark 1996). There exist both symbiotic and free-living diazotrophs that can reduce N_2 to ammonia (NH_3) in almost all habitats. The reaction is inhibited by oxygen and

by the presence of nitrate (NO_3^-). Nitrogen fixation is mediated by a number of genes including those that code for the nitrogenase enzyme, which exists in a complex with two Fe-S proteins. *nif*H is the structural gene for dinitrogenase reductase, while additional *nif* genes function as transcriptional regulators, code for cofactors, or are involved in electron transport. Production of the nitrogenase enzyme is inhibited by the presence of its product, NH_3. Overall, nitrogen fixation is not favoured in the presence of available N. An increase in mineralization with warming could stall this process in the arctic. Once the NH_3 is transformed to NH_4^+, it is available for microbial growth and plant uptake, becomes adsorbed to minerals and organic matter in the soil, or is lost due to leaching or as a volatile gas. Additionally, NH_4^+ is used as an energy source by nitrifying bacteria (Paul and Clark 1996).

Nitrification is detectable in arctic soils, and there are different degrees of nitrate use by each plant community (Paul and Clark 1996, Nadelhoffer *et al.* 1992). It is a temperature sensitive process that likely occurs only in the growing season and that could be greatly affected by potential soil warming (Paul and Clark, 1996). Direct increases in nitrification due to higher temperatures may be in addition to the increases attributed to a higher availability of substrate if warming also promotes mineralization (Nadelhoffer *et al.* 1992). Nadelhoffer *et al.* (1992) predicted that arctic ecosystems with an intermediate moisture regime will see the greatest increases in nitrification with warming; the drier systems lack the

deep OM layer required for large changes in the soil environment, and biological processes at the wetter sites are limited by anaerobic conditions.

The nitrification pathway from ammonium (NH_4^+) to nitrate (NO_3^-) is mediated by specific microbes; NO_3^- is preferred by plants, but is easily lost due to high solubility in water (Paul and Clark 1996, Prescott *et al.* 1999). This process is accomplished in two stages primarily by chemoautotrophic aerobes that oxidize NH_4^+ to hydroxylamine or that oxidize nitrite (NO_2) to NO_3^-. These low energy-yielding reactions are mediated by the enzymes ammonium monooxygenase, hydroxylamine oxidoreductase, and nitrite dehydrogenase (Paul and Clark 1996). The ammonium oxidizers (Nitroso- spp.) are members of the beta or gamma proteobacteria while the nitrite oxidizers (Nitro- spp.) occupy the alpha proteobacterial group (Prescott *et al.* 1999). Nitrification is sensitive to pH and does not occur below pH 4.5 in agricultural soils; forest soils that harbor heterotrophic nitrifiers can support nitrification even in slightly acidic conditions. This process requires an aerobic, mesic environment and is slow below 5°C (Paul and Clark 1996).

Nitrate can be reduced by other members of the Proteobacteria (*Rhizobium*, *Alcaligenes*, *Pseudomonas*) and Archaea (*Haloarcula*) (Prescott *et al.* 1999), and lost as environmentally detrimental gaseous byproducts of denitrification (Paul and Clark 1996). Denitrification is anaerobic respiration that uses oxidized inorganic forms of nitrogen as electron acceptors (Paul and Clark 1996, Prescott

et al. 1999). Steps in this pathway are mediated by the enzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase; the final product is nitrogen gas (N_2) (Paul and Clark 1996). This process is inhibited in an oxygenated and/or acidic environment and has been observed in some diazotrophs but is temporally separated from nitrogen fixation because the enzyme nitrogenase is repressed by NO_3^-. Denitrification has also been observed in some heterotrophic nitrifiers; the process is spatially separated but can occur at the same time as nitrification. If carbon supply is limited, incomplete reduction of NO_3^- can occur with a resulting accumulation of the nitrogen oxide gases. Denitrification potential decreases linearly with soil temperatures below 15°C (to a minimum at 5°C), and is optimal in oxygen limited soils with pH 6-8 (not detected below pH 4) (Paul and Clark 1996).

Nitrification and denitrification may be closely associated, especially at aerobic/anaerobic interfaces where the nitrification product, NO_3^- is readily available for reduction (Nicolaisen *et al.* 2004). Gaseous losses of nitrogen have been attributed to these coupled cycles, but Nicolaisen *et al.* (2004) suggest that plant roots are strong competitors for available nitrogen, so that in their presence loss to the atmosphere is minimized.

1.5 Microbial communities

Nitrogen fixers are represented across most prokaryotic groups, and include organotrophic and phototrophic, aerobic and anaerobic microorganisms that possess the enzyme nitrogenase (Paul and Clark 1996, Zehr *et al.* 2003). The free-living aerobes such as *Azotobacter* and *Beijerinckia* can be found on the surface of roots and in the adjacent soil. Their high rates of respiration are one strategy to protect the nitrogenase enzyme from oxygen. The microaerophiles (*Azospirillum, Bacillus*) and anaerobes (*Clostridium, Desulfovibrio)* already occupy the anoxic environment required by this enzyme. There are also a significant proportion of diazotrophic cyanobacteria (*Nostoc, Anabaena*) above and below the soil surface that contribute greatly to nitrogen fixation in the arctic (Chapin and Bledsoe 1992, Liengen 1999, Paul and Clark 1996). Other aquatic species include the nitrogen fixing green and purple sulfur bacteria. Symbiotic actinomycetes such as *Frankia* fix nitrogen in association with the arctic plant *Dryas* (Paul and Clark 1996).

Based upon modes of nutrition, aerobic nitrifiers should be found at and just below the soil surface, where litter and humus are mineralized providing net available NH_4^+ (Paul and Clark 1996). It follows that the heterotrophic denitrifiers would be found at a depth where anaerobic conditions prevail, and where there is a carbon source, for example, below the rhizosphere (Paul and Clark 1996). Nicolaisen *et al.* (2004) confirm that abundance and activity of nitrifiers follows a

distribution based upon the presence of O_2 and NH_4^+ in the soil. By separating their samples into surface, bulk, or rhizosphere zones, they noted highest numbers and nitrification potential in the surface soil (2-3 cm deep) with lowest numbers and nitrification potential in the bulk soil (5-6 cm deep). The authors note that by overlooking the differences between these soil depths, the surface may not be appropriately recognized as an important nitrification site. In addition, molecular techniques can detect nitrifiers in deeper samples although they may simply be in a dormant state due to unfavourable anoxic conditions. Despite these findings, differences in nitrifier community structure were not apparent across the three zones; in fact, diversity was low overall when compared to other soil environments (Nicolaisen *et al.* 2004). Avrahami and colleagues (2002), also show that increased NH_4^+ availability promotes an increase in nitrous oxide production, especially via nitrification, without a corresponding community shift in the nitrifier community. However, when the denitrifier population was examined with Terminal Restriction Fragment Length Polymorphisms (T-RFLP) of the *nirK* gene, changes in diversity were detected that appeared to mirror changes in nitrous oxide (N_2O) contribution by the different microbial communities (Avrahami *et al.* 2002).

Further studies by the same group showed a shift in the ammonia oxidizer community with temperature manipulations in the lab (Avrahami and Conrad 2003). Their previous measurements of increased nitrifier activity levels with fertilizer additions could not be attributed to a community shift; there may instead

have been a physiological adaptation of the existing community. Avrahami and Conrad (2003) once again measured nitrification activity in fertilized treatments with different soil temperatures, moisture levels, and pH; the lowest activity was noted in the coolest soils. Using the gene target *amo*A, the researchers showed that acidic soils had a higher diversity of ammonium oxidizers, and that both acidic and alkaline soils experienced a change in community structure with temperature manipulations. Most importantly, the researchers concluded that temperature was selecting for different ammonium oxidizing communities, and that long term temperature changes will result in changes to the community structure of these organisms in the natural soil environment (Avrahami and Conrad 2003).

1.6 Genetic diversity versus physiological function

The link between changes in microbial community structure, measured by changes in gene diversity, and corresponding changes in the processes mediated by these communities has not been satisfactorily established. Community shifts that do not affect microbial activity have been documented (Avrahami and Conrad 2003, Deslippe 2004) and changes in physiological function have been noted without affecting diversity at the genetic level (Gomez *et al.* 2004, Nicolaisen *et al.* 2004, Rich and Myrold 2004). It has been suggested that biotic and abiotic environmental factors can structure the genetic composition of a system, but that gene selection is not the only driver of microbial

diversity (Zehr *et al.* 2003). Zehr *et al.* (2003) explain that a habitat not limited by nitrogen availability should not select for the functional genes required for nitrogen fixation. In such a habitat, the diversity of these genes should diminish, but the opposite has been documented, suggesting that function and diversity are not related in this system. Alternately, Rich *et al.* (2003) found a significant relationship between denitrifier community composition and denitrification activity on the basis of vegetation type. The proportional abundance of at least two dominant terminal restriction fragments clearly differed in a forested area of low denitrification versus a meadow with high activity (Rich *et al.* 2003). Further studies by the same group confirmed unique *nos*Z TRF communities at each of three distinct sites, but denitrifying activity measurements did not vary with microbial community structure (Rich and Myrold 2004). PCR plus denaturing gradient gel electrophoresis (DGGE) also failed to detect denitrifier community shifts corresponding to increasing N_2O, but the same genetic marker revealed changes in diversity associated with the rise in N_2O when gene expression was measured by real time PCR (RT-PCR) (Sharma *et al.* 2006). Although changes in the distribution of functional gene markers may not yet reliably predict changes in physiological function, techniques that measure the presence and/or relative abundance of functional genes are ideal for assessing microbial community structure (Tiedje *et al.* 1999, Zehr *et al.* 2003).

1.7 Molecular techniques

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a molecular technique that allows researchers to examine the diversity of the microbial genes associated with important soil processes (Tiedje *et al.* 1999). The procedure uses polymerase chain reaction (PCR) amplification with fluorescently labeled primers and subsequent restriction digests of the PCR product to generate terminal restriction fragments (TRFs) that are representative of unique taxonomic units. The presence and relative abundance of these fragments are interpreted by an automated sequencer, and their patterns can be used to describe differences in gene distribution and richness (Dunbar *et al.* 2000, Tiedje *et al.* 1999). This assessment of microbial community structure can be used to compare the differences in potential gene function based upon larger scale environmental change especially where species diversity is low or moderate (Engebretson and Moyer 2003). The technique is rapid and repeatable, but may overestimate similarity and thus underestimate diversity when compared to cloning and sequencing methods (Dunbar *et al.* 2000). It is highly dependent on the choice of restriction enzyme; accurate measures of diversity require that all unique restriction sites are identified (Dunbar *et al.* 2000, Engebretson and Moyer 2003).

Chapter 2. Detection of shifts in microbial functional gene (*nosZ* and *nifH*) distribution due to long term warming of a high arctic soil.

2.1 Introduction

Climate change is expected to disproportionately affect arctic and antarctic latitudes (Hassol 2004, Maxwell *et al.* 1992). Positive feedbacks due to changes to polar weather patterns and ecosystems will further affect global climate. Current climate change models assume a doubling of atmospheric carbon dioxide (CO_2) concentration in less than fifty years. The effects are dramatic: within 100 years, annual arctic temperature may be 3-5 °C higher, with winter temperatures as much as 4-7 °C higher over land than they are currently (Hassol 2004, Maxwell *et al.* 1992). Most researchers agree that overall annual global precipitation will increase, and, like temperature, will be greater at the poles. Contradictions arise regarding net soil moisture; some suggest an increase due to melting of the permafrost layer and earlier snowmelt, while others hint at a decrease due to better drainage and increased evaporative losses (Hassol 2004, Kane *et al.* 1992, Maxwell *et al.* 1992). Increased surface temperature resulting in heat transfer to the soil via conduction has also been assumed (Kane *et al.* 1992).

There are coastal lowlands in the high arctic with greater productivity and species diversity due to different temperature and moisture regimes than the surrounding desert-like plateaus (Freedman *et al.* 1994). These unique ecosystems provide excellent study sites for the predicted changes due to global warming because of

seasonal extremes in solar energy and precipitation, and because these areas are expected to experience the greatest transitions (Freedman *et al.* 1994, Maxwell *et al.* 1992). The research site at Alexandra Fiord is on the northern side of Johan Peninsula, on the eastern coast of Ellesmere Island, Nunavut (Freedman *et al.* 1994). The study area encompasses a lowland outwash plain, bordered by ocean to the north and glacier to the south, and by the cliffs of an upland plateau, up to 750 m higher on the east and west. Polyethylene open-topped chambers (OTCs) have been established across various gradients at this site, and many studies have taken place over the last decade. Warming due to these small greenhouses has been shown to increase plant productivity and create community shifts compared to adjacent control plots on wet, moist, and dry lowland, and on granitic and dolomitic upland sites (Freedman *et al.* 1994, Rolph 2003, Walker *et al.* 2006).

All lowland sites and both the granitic and dolomitic upland sites are considered nutrient (especially nitrogen) limited, but even slight increases in both temperature and precipitation can lead to deeper active layers, higher rates of chemical transformations and ultimately, more nutrient availability (Berendse and Jonasson 1992, Muc *et al.* 1994). Changes in soil moisture status will impact microbial processes such as decomposition in dry, moist, and wet arctic ecosystems; the resulting combination of moisture and temperature will dictate organic matter turnover rates, and ultimately ammonium (NH_4^+) availability via mineralization (Paul and Clark 1996, Nadelhoffer *et al.* 1992). It is possible that

future arctic environments will be less nitrogen limited due to a predicted rise in nitrogen fixation with increased enzyme activity and levels of carbon dioxide (Chapin and Bledsoe 1992) but in general, nitrogen fixation is not favoured in the presence of available nitrogen (N) therefore an increase in mineralization with warming could stall this process in the arctic (Paul and Clark 1996).

Direct increases in nitrification due to higher temperatures may be in addition to the increases attributed to a higher availability of substrate if warming does indeed promote mineralization (Nadelhoffer *et al.* 1992). Nitrification and denitrification may be closely associated, especially at aerobic/anaerobic interfaces where the nitrification product, nitrate (NO_3^-) is readily available for reduction (Nicolaisen *et al.* 2004). If soil warming is favourable for nitrification, it may also stimulate denitrification and subsequent nitrous oxide (N_2O) production (Paul and Clark 1996). We know that experimental nutrient amendments increase the rates of N_2O production, but it still has not been confirmed if this is correlated with the genetic structure of denitrifier communities (Rich and Myrold 2004). Additionally, a habitat with excess nitrogen should not select for the functional genes required for nitrogen fixation (Zehr *et al.* 2003) but the diversity of the nitrogen fixing community has not been shown to diminish in such cases (Piceno and Lovell 2000a).

The objective of this study was to investigate changes in the frequency, relative abundance and richness of the functional genes *nosZ* and *nifH* as markers for denitrification and nitrogen fixation respectively. We were looking for shifts in the denitrifying and nitrogen fixing soil microbial communities from sites with different dominant plant communities, on different parent material and across different moisture regimes after a thirteen year warming experiment in the High Arctic. Our null hypothesis was that long term warming treatments would not alter microbial community structure.

2.2. Methods

2.2.1. Site description

The research site at Alexandra Fiord is on the northern side of Johan Peninsula, on the East coast of Ellesmere Island, Nunavut (78°53' N, 75°55' W) (Freedman *et al.* 1994). The study area encompasses a lowland outwash plain, bordered by ocean to the north and glacier to the south, and by the cliffs of an upland plateau, up to 750 m higher on the east and west.

The topography of the area leads to warmer temperatures and greater accumulations of water in the lowland plain than the surrounding landscape; this qualifies the lowland as a true polar oasis (Freedman *et al.* 1994). There is a relatively large amount of organic matter in the young soils, and diverse vegetation including cushion plants and dwarf shrubs. In summer, the dark soil surface results in a low albedo which contributes to higher air and soil temperatures at this site than those measured at the next closest weather stations in Eureka and Resolute (Labine 1994).

The regoliths of the upland area are drier overall than the lower site, but support some of the same plant species on both granitic and dolomitic parent rock (Freedman *et al.* 1994). During the growing season this area experiences below zero air temperatures and less absorbed solar radiation than the coastal lowland (Labine 1994).

Five distinct sites are investigated in this study (Figure 2). They differ primarily by dominant plant community, and each corresponds to a particular soil moisture gradient and/or soil parent material (Muc *et al.* 1989).

All soils at the three lowland sites are derived from granitic material. The Sedge Meadow site (SM) is characterized as the wettest (hydric) with a thick organic layer over mineral soil and a pH range of 6.6 in surface soils to 5.9 in deeper layers. It is dominated by Sedge, Cushion Plant, and Dwarf Shrub species that include *Carex stans, Polygonum viviparum* and *Vaccinium uliginosum* plus hummocks of *Salix arctica* and *Dryas integrifolia.* The Cassiope Heath site (CH) has an average of 3-5 cm of organic soil over coarse mineral soil with a pH range of 4.9 – 5.4; the site is described as hydric-mesic. Cushion Plant and Dwarf Shrub species at this location include *Cassiope tetragona,* which dominates, plus *S. arctica, D. integrifolia,* and *Saxifraga oppositifolia.* The driest (mesic-xeric) lowland site is Riverside Willow (RW). Sandy mineral soils here range in pH from 5.2 to 4.6, and support the greatest plant diversity of all sites. Deciduous dwarf shrubs and graminoid species are present, primarily *Salix arctica* and including *Festuca brachyphylla.*

The Upland Granite (UG) and Upland Dolomite (UD) study sites are distinguished by the origin of their mineral soils and this is reflected in their soil pH. UG has an acidic pH range of 4.9 – 5.5, while the alkaline UD has an average pH of 7.9. Both sites are xeric, though UD is somewhat drier, and are dominated by the

deciduous dwarf and semi-evergreen shrubs *S. arctica* and *Dryas integrifolia* (Klady 2006, personal correspondence, Muc *et al.* 1989). Site data collected in previous experiments at this study area are summarized in Table 1.

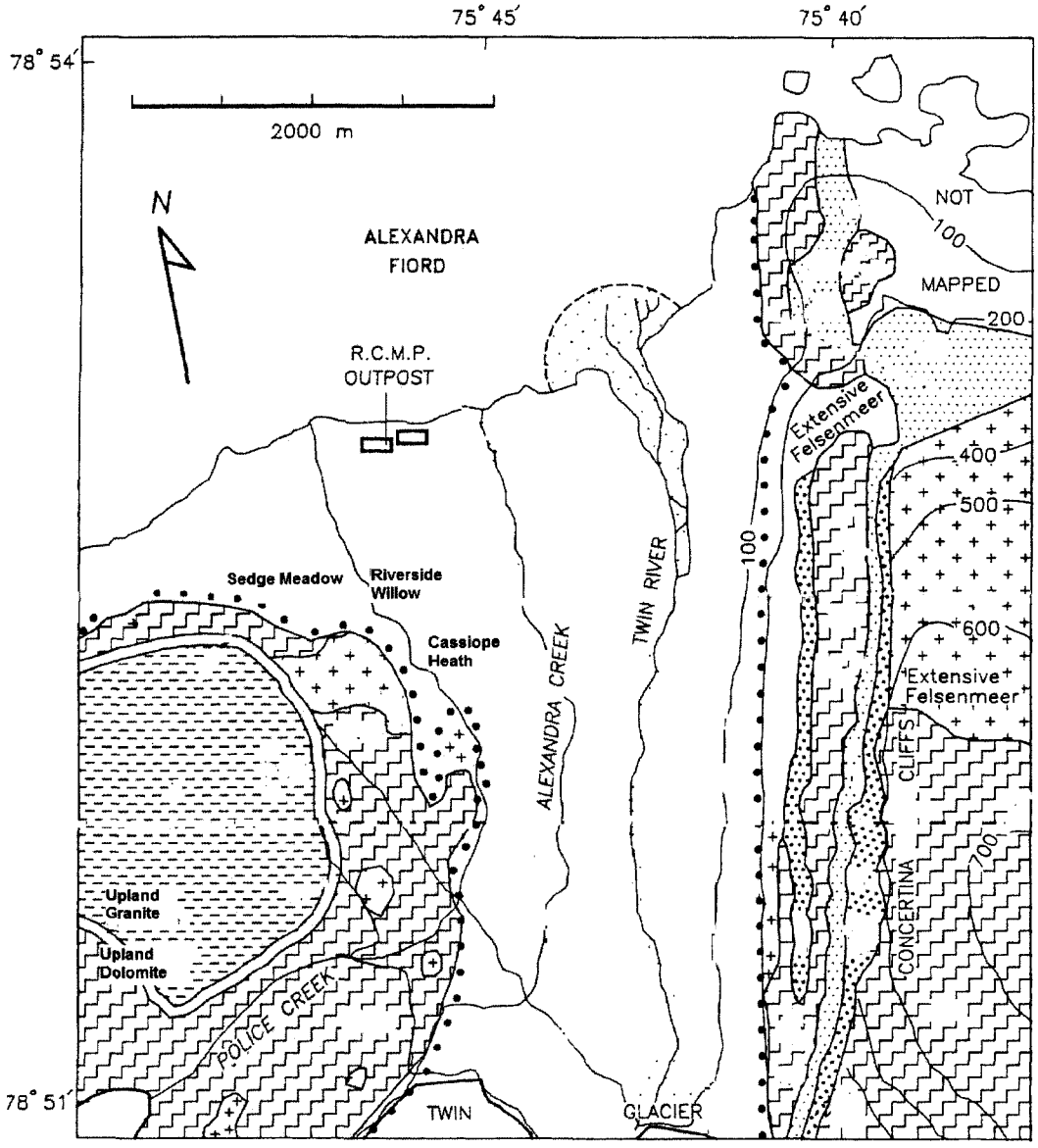

Figure 2: Layout of the Alexandra Fiord Study area showing the three lowland sites (Sedge Meadow, Cassiope Heath, and Riverside Willow) and the two upland sites (Upland Granite and Upland Dolomite). Contour intervals are 100 m (modified from Sterenberg and Stone 1994).

Site	Treatment	pH[a]	SWC[b]	Air temp (°C)[c]	Soil temp (°C)[c]
SM	Control	6.3	86.3	8.35	6.40
	OTC		91.3	9.81	7.05
CH	Control	5.2	37.6	8.61	9.16
	OTC		40.0	8.90	9.36
RW	Control	4.9	39.6	8.48	9.19
	OTC		39.0	9.55	9.66
UG	Control	5.2	0.120	n/a	n/a
	OTC		0.125		
UD	Control	7.9	0.083	n/a	n/a
	OTC		0.070		

[a] pH average over upper and lower soil samples (Klady, personal correspondence)
[b] approximate volumetric SWC (%) (lowland) and gravimetric SWC (g H_20/g soil) (upland) averaged over growing season (data compiled from Rolph 2003)
[c] air temperature at +10 cm and soil temperature at -10 cm (SM) or -2 cm (CH and RW) averaged over growing season (Rolph 2003)

Table 1: Individual site data collected during other warming experiments at Alexandra Fiord include soil pH, soil water content (SWC) and both air and soil temperatures.

2.2.2. Experimental design

Open Topped Chambers (OTCs)

All lowland treatments plots had 1.8m^2 hexagonal transparent fiberglass chambers with 0.5m high inclined sides in place where limited non-destructive sampling had occurred since their installation in 1992 (Rolph 2003). OTCs were installed at the upland site the following year, but these sites were faced with an increased exposure to wind and some of the treatment plots were missing a warming chamber, while others had been replaced with a smaller version of the original OTC in 2002 (Klady, personal correspondence June 2006). These structures remained in place throughout the year and did not cause a significant

difference in SWC when this factor was measured in 2001 (Rolph 2003). Based upon data averaged over the 2001 growing season, OTCs at the lowland sites increased air temperatures by 0.3 to 1.5°C and soil temperatures up to 0.7°C at -10 cm (Table 1).

Control plots

For all lowland sites, adjacent control plots were 25 paces from their associated OTC in a direction perpendicular to the overall site layout in order to preserve the original random location of each treatment plot. For both upland sites, the same method was used, but was limited to 10 paces due to size constraints of the area.

Randomization

Random sampling was accomplished with a random numbers table plus a 50 cm X 50 cm quadrat. The quadrat was always placed as close to the center of each OTC as was practical, or was dropped at 25 or 10 paces from the OTC for every control plot. The quadrat was divided into 5 cm X 5 cm squares that easily accommodated the soil corer, and the numbers table was used to choose rows and columns for sampling.

Soil sampling

Four OTC pairs (one OTC plus one adjacent control plot) were sampled at each of the five sites: three on the lowland (Sedge Meadow, Cassiope Heath and Riverside Willow) and two on the upland (Granitic and Dolomitic). Sampling

occurred once at each site over a period of peak plant growth (Rolph 2003) from July 18 to 27, 2004. Six 2 cm diameter soil cores were taken from each OTC and from each of the corresponding control plots. A 45 cm long soil corer was used, and samples were recovered from the top 5 cm (upper) and the bottom 5 cm (lower) layers based upon the depth of the core. This corer adequately represented all sites as the active layer never extended beyond its reach. Upper samples were always taken from the top 0-5 cm of the soil core, while the average depths of lower samples were as follows: Sedge Meadow 39-44 cm, Cassiope Heath 38-43 cm, Riverside Willow 34-39 cm, Upland Granite30-35 cm, and Upland Dolomite 8-13 cm. Cores that were not at least 10cm deep were rejected so that a separation could always be made between the top and bottom 5 cms. Approximately 1 g of soil was taken from each of the six replicates at the two different depths. This yielded 12 x 1 g soil samples from each OTC plus 12 x 1 g soil samples from each control plot. The twenty-four samples from each OTC pair, for four pairs at each of five treatment sites, resulted in 480 x 1 g samples frozen for transport back to UNBC for DNA extraction.

2.2.3. Preservation and transport

The 1 g samples remained frozen on site in an underground 'permafrost refrigerator' in 2 mL microcentrifuge tubes and in sealed airtight bags until transport to UNBC. Every attempt was made to keep the samples frozen at -20°C during transport and upon return to UNBC until ready for DNA extraction.

2.2.4. Field measurements

Air and soil temperature, soil water content (SWC), and soil chemical analysis were to be provided by Dr. GHR Henry (Alexandra Fiord ITEX Research Site Supervisor, UBC Department of Geography) upon return to UBC in the fall of 2004. Additional soil cores were collected for this purpose from representative plots across all sites during the 2004 growing season. This information was to include (at least) air and soil temperature at +10, -2 and -10 cm from data collected by thermocouples and data loggers at each site, along with SWC, pH, and C:N. Data from the 2004 growing season is currently being compiled and is not yet available as of June 2006 (Klady, personal correspondence June 2006).

Data from throughout the 2001 growing season was collected as follows (Rolph 2003):

Soil and air temperature at the Sedge Meadow site were measured at -10cm and +10cm, respectively. Hobo® Pro Temperature loggers (H8, Onset Computer Corp. MA, USA) with thermistors connected to Pocket Data Loggers (XR220, Pace Scientific, NC, USA) were employed for this purpose. Thermocouples connected to data loggers (CR-10, Campbell Scientific Inc., UT, USA) were used to collect soil and air temperature data at -10 cm, -2 cm, and +10 cm at both the Cassiope Heath and Riverside Willow sites. Volumetric SWC was measured with Hydrosense™ probes (Campbell Scientific Inc., UT, USA) at lowland sites while gravimetric SWC was assessed by soil cores from upland sites. Measurements were made four times throughout the season at both control and OTCs with the

exception of the upland sites; in this case OTCs were sampled only once at the end of the season.

2.2.5. DNA extraction and PCR

DNA extractions were performed with the MoBio UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories, Inc., CA, USA) according to the "Alternative Protocol for maximum yields". Spectrophotometric analysis of extractions revealed final DNA concentrations of 50-150 ng/µl.

Each extraction was amplified with two different primer pairs. Functional genes targeted included those that code for the denitrification enzyme nitrous oxide reductase (*nos*Z), and for the nitrogen fixing enzyme nitrogenase (*nif*H). Degenerate primer pairs were designed, tested and optimized for the genes *nos*Z (Throbäck *et al.* 2004), and *nif*H (Deslippe 2004).

The half-nested *nos*Z amplification utilized the primers nosZ-F (5'-CG(C/T) TGT TC(A/C) TCG ACA GCC AG-3') (Kloos et al. 2001 [In Throbäck *et al.* 2004]) and nosZ1622R (5'-CGC (G/A)A(C/G) GGC AA(G/C) AAG GT(G/C) CG-3') (Throbäck *et al.* 2004) for the primary amplification. The secondary reverse primer Nos1773R (5'-AAC GA(A/C/G) CAG (T/C)TG ATC GA(T/C) AT-3') (Throbäck *et al.* 2004) was labeled with Light Sabre Blue (D4) dye (Synthegen, LLC). Each 30µl PCR reaction contained 3 µL 1:10 dilutions of genomic DNA, 1X PCR Buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.04 µM of each primer, and 0.75 U

Platinum Taq DNA Polymerase (Invitrogen). The secondary PCR mix differed only in $MgCl_2$ concentration (2.125 mM). Thermocycler conditions were the same for both reactions: a 2 minute denaturation step at 94°C was followed by 35 cycles of denaturing, annealing and extension at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute respectively. The final extension required 10 minutes at 72°C.

The half-nested *nif*H protocol used Nh21F (5'-GCIWTITAYGGNAARGGNGG-3') and WidNhR (5'-GCRTAIABNGCCATCATYTC-3') for the primary PCR reaction (Widmer *et al.* 1999) and Nh428R (5'-CCRCCRCANACMACGTC-3') for the second amplification (Deslippe 2004)(sequences follow standard IUPAC notation for mixed bases). The reverse primer Nh428R was labeled with Synthegen Light Sabre Green (D3) dye. Each 31.2 µl PCR reaction contained 4.5 µL 1:10 dilutions of genomic DNA, 1X PCR Buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.04 µM of each primer, and 0.75 U Platinum Taq DNA Polymerase (Invitrogen). Thermocycler conditions were the same for both reactions: a 1 minute denaturation step at 94°C was followed by 35 cycles of denaturing, annealing and extension at 94°C for 45 seconds, 53°C for 45 seconds, and 72°C for 1 minute 30 seconds respectively. The final extension required 10 minutes at 72°C.

PCR product success and quality was assessed by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. Bands of expected size (approximately 250bp for *nos*Z and approximately 400bp for *nif*H)

were cleaned via ethanol precipitation and then resuspended in pH 8.0 Tris-EDTA Buffer.

2.2.6. T-RFLP

Restriction enzymes used included *Hha*I for *nos*Z product and *Mbo*I for *nif*H plus corresponding REACT 2 buffer for both endonucleases (Invitrogen). Enzymes were selected based upon number of restriction sites targeted (and therefore fragments generated); a series of endonucleases were tested on replicate samples and those that identified the greatest amount of variation were chosen. For each reaction, 6 µL of PCR product was digested with 2.5 U enzyme and 1X buffer. Digests were incubated at 37°C for at least 5 hours and the reactions were terminated at 65°C for 10 minutes. Digested fragments were desalted by ethanol precipitation and resuspended in formamide. Fragments were prepared for analysis as suggested by the manufacturer for the Beckman-Coulter CEQ™ 8000 Fragment Analysis System (Beckman-Coulter Inc.) for 40µl non-multiplexed samples, although resuspended fragments were not diluted 1:10 prior to loading. For each reaction, 2.5 µL of dye-labeled, digested, desalted product was combined with 37.0 µL of Sample Loading Solution (SLS) and 0.5 µL of 400 bp size standard.

Fragments were binned and analyzed in the AFLP program of the CEQ™ 8000 Sequencer (Beckman-Coulter Inc.). Analysis parameters were as per the 400 size standard cubic model with minimum relative peak height set at 1% and a bin

width of 3 bp. At least four of six field replicate samples from each of two soil depths were analyzed individually for all four treatment and all four control plots from all five sites. Gene community profiles were constructed using only peak heights generated by the sequencer that passed analysis. These peaks represented distinct terminal restriction fragments (TRFs) which in turn corresponded to unique genotypes. Samples that failed due to contamination during fragment analysis or undetected size standard were deleted so that they were not counted with zero-activity samples.

TRF frequency was determined for each sample by averaging the binary data for all successful field replicates. For example, if a TRF was present in 3 of 6 sample replicates, its frequency was designated 0.50 for that sample. This made it possible to compare samples with only 4 successful field replicates after PCR to those with 5 or 6. In order to establish the relative abundance of TRFs, the fluorescent signal strength of each peak was relativized to total peak area for each successful field replicate. Once the relative abundance of TRFs was determined for each sample rep, the average was calculated to determine the relative abundance of TRFs for that sample.

2.2.7. Statistical analysis

Community composition was investigated graphically with Nonmetric Multidimensional Scaling (NMS) calculated on the basis of a Sørensen distance

measure. All ordinations were run with PC-ORD 4.0 software (McCune and Mefford 1999) using random starting coordinates. A stepwise reduction in dimensionality was used to minimize stress and 40 runs with real data were accomplished in autopilot mode. Stress is defined as excellent below 5, good below 10, and fair above ten; caution is advised when interpreting ordinations with values approaching 20 (McCune and Grace 2002).

Any differences observed in frequency and/or abundance of TRFs were tested statistically with nested permutational multivariate ANOVA (PERMANOVA) in order to examine the effects of Site, Treatment, and Depth (Anderson 2005). A large number of unique permutable units allowed us to establish significance using permutation P-values versus Monte Carlo P-values.

Genotypic richness was assessed by comparing differences in the number of TRFs at each site, depth and treatment (Dunbar *et al.* 2000). Each TRF represents a unique genotype so that an increase or decrease in these numbers reflects an increase or decrease in genotype richness (Tiedje *et al.* 1999). Nested ANOVA (Statistica 6.0) was used to detect overall significant differences between sites and due to depth or treatment at all sites. Once this was determined, one-way ANOVA was then used to test statistical significance between sites pairwise, and to investigate differences due to depth or treatment unique to each site. For all statistical tests, a significance level of $p < 0.05$ was accepted.

2.3. Results

2.3.1 Gene frequency and relative abundance

Site effect

TRF frequency and relative abundance was shown to differ overall by site in NMS ordinations of both *nosZ* and *nifH* functional genes. Additionally, PERMANOVA suggested significant overall support for an effect of the factor "Site" upon examining the frequency data of both functional genes (p=0.0001). PERMANOVA analysis also confirmed a site effect for the relative abundance data of both *nosZ* (p=0.0001) and *nifH* (p=0.0012).

More specifically, PERMANOVA showed significant differences in *nosZ* TRF frequency between the Sedge Meadow and all other sites: SM and CH (p=0.0269), SM and RW (p=0.0276), SM and UG (p=0.0274), and SM and UD (p=0.0307). Additionally, dissimilarities were detected between CH and both upland sites (UG p=0.0287 and UD p=0.0281) plus RW and both upland sites (UG p=0.0305 and UD p=0.0301). Interestingly, the upland sites also differed from each other (p=0.0285). These differences are illustrated in Figure 3a. An NMS graph (Figure 4) shows *nosZ* frequency data grouping clearly by Site. It illustrates the unique nature of SM, and the similarities between CH and RW. The recommended three dimensional solution required 400 iterations and resulted in a good final stress of 9.07458 and a final instability of 0.00411. Together Axes 1 and 3 explain 72.4% of the variance, while Axis 2 adds 23.1% (cumulative r^2=0.954).

Specific differences in *nif*H TRF frequency were detected by PERMANOVA between SM and CH (p=0.0265), SM and UD (p=0.0281), CH and RW (p=0.0281), CH and UD (p=0.0285), RW and UG (p=0.0279) and finally RW and UD (p=0.0289). These dissimilarities are illustrated more clearly in Figure 3b. Figure 5 shows *nif*H frequency data grouping generally by Site for RW and UD, with weak differentiation of the other three sites. This illustrates the unique nature of UD, but that the other sites are not enormously different from each other. The recommended two dimensional NMS solution required 400 iterations and resulted in a fair final stress of 13.543 and a final instability of 0.00401. Together these two axes explain 92.2% of the variance but the image should be interpreted cautiously due to high stress and instability values.

Figures 6 and 7 show only the top 20 most frequent TRFs over all sites, and how they are distributed. The former shows differences in *nos*Z TRF distribution between all fives sites, while the latter reflects the same for *nif*H.

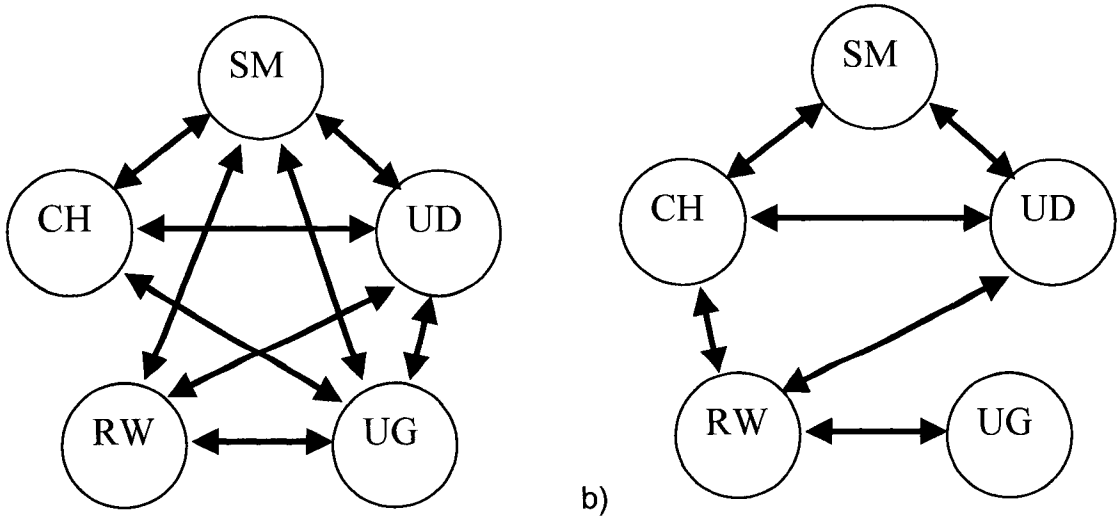

Figure 3: Arrows indicate significant differences in the frequency of *nos*Z (a) and *nif*H (b) TRFs between sites.

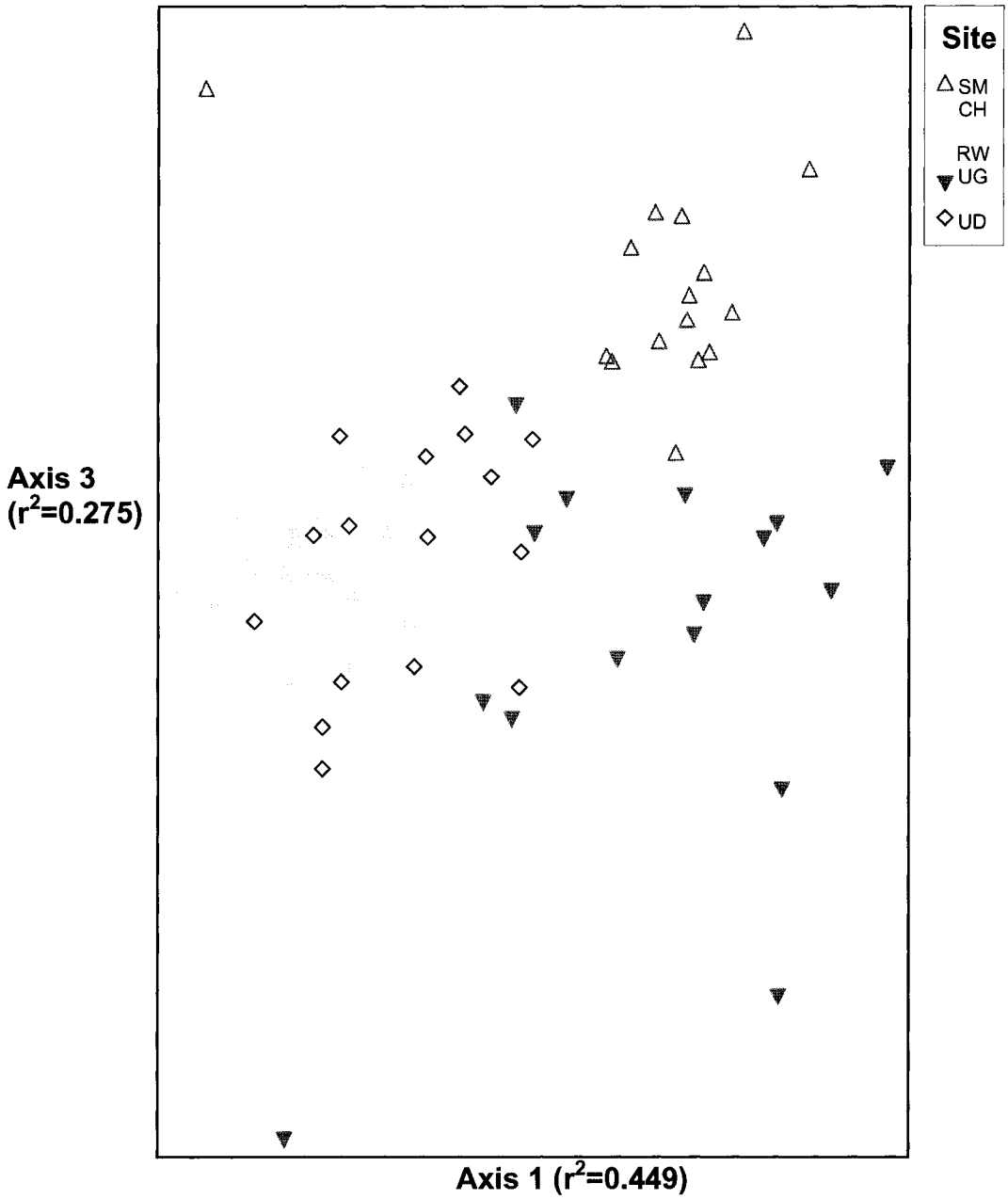

Figure 4: NMS plot of *nos*Z TRF frequency by Site p=0.0196. Sites are: Sedge Meadow (SM), Cassiope Heath (CH), Riverside Willow (RW), Upland Granite (UG), and Upland Dolomite (UD).

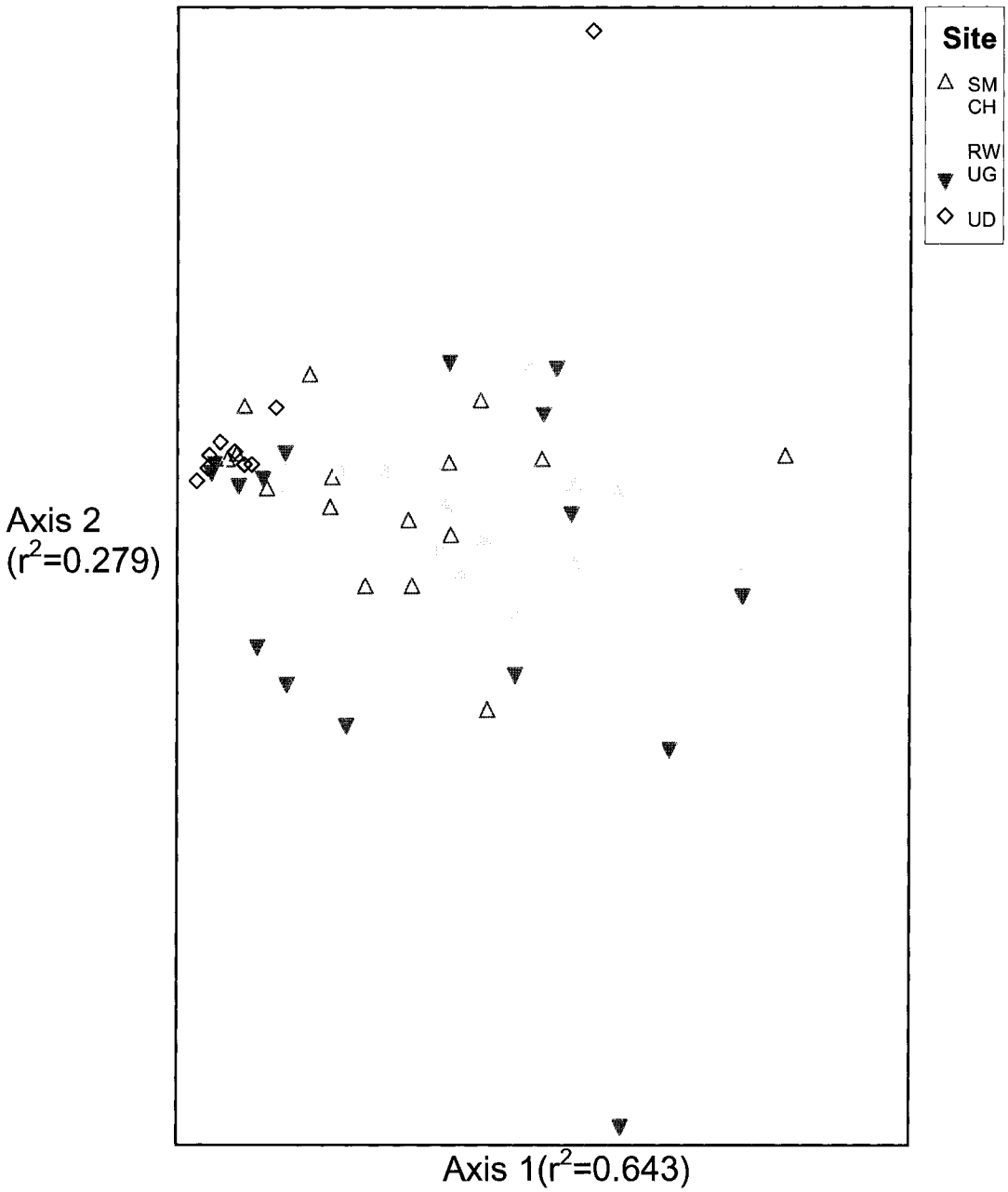

Figure 5: NMS plot of *nif*H TRF frequency by Site p=0.0196. Sites are: Sedge Meadow (SM), Cassiope Heath (CH), Riverside Willow (RW), Upland Granite (UG), and Upland Dolomite (UD).

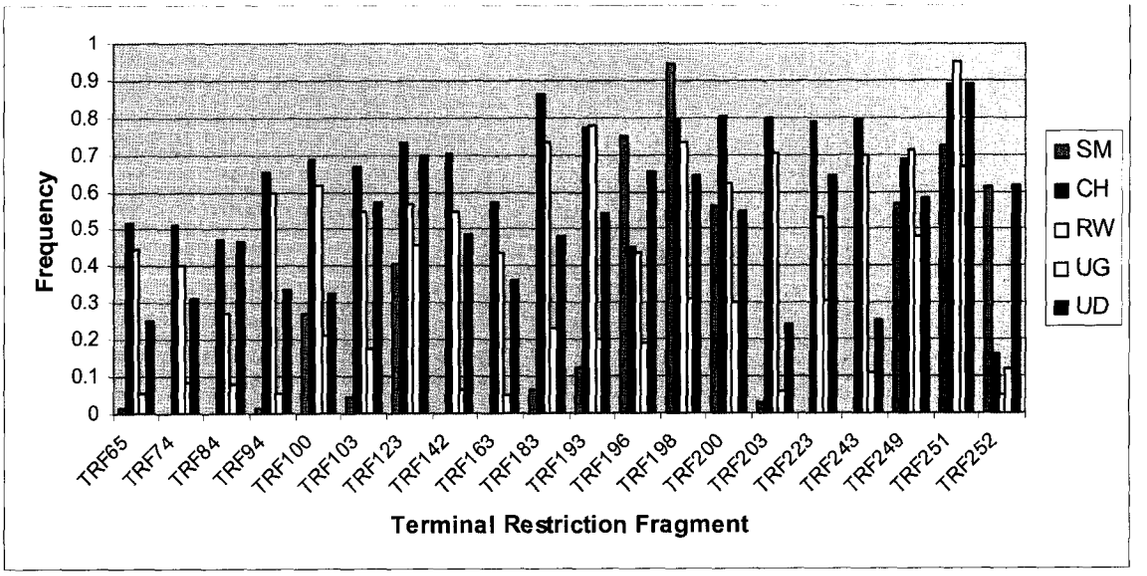

Figure 6: Distribution of the top 20 most frequent *nosZ* TRFs over all Sites. Sites are: Sedge Meadow (SM), Cassiope Heath (CH), Riverside Willow (RW), Upland Granite (UG), and Upland Dolomite (UD).

Sedge Meadow was defined by the presence of a few dominant TRFs; only 6 *nosZ* TRFs showed a frequency greater than 0.50 (Figure 6). These are present in two main clusters: 196/198/200 and 249/251/252. This site is structured by TRF 198 (0.943), TRF 196 (0.749), TRF 251 (0.724), TRF 252 (0.611), TRF 249 (0.568), and TRF 200 (0.565). A greater number of distinct TRFs were present at Cassiope Heath and Riverside Willow and many were shared between both sites. The most frequent CH TRFs are 251 (0.889) 183 (0.866), 200 (0.802), 203 (0.801), and 198 (0.795). These are similar to those at RW: 251 (0.948), 193 (0.780), 183 and 198 (both 0.734), and 249 (0.715). TRF 251 dominated both upland sites as well (UG, 0.669 and UD, 0.889) but all other UG TRFs were far less frequent: 249 (0.481), 123 (0.455), 198 (0.310), and 223 (0.306). UD also contained TRFs 123 (0.700), 196 (0.651), 198 and 223 (both 0.643).

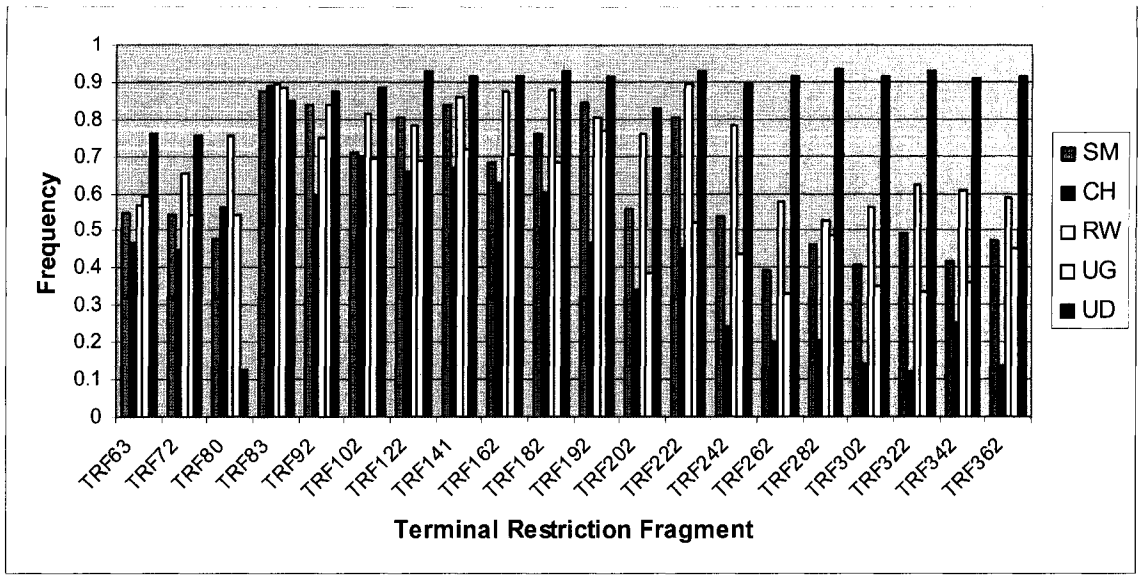

Figure 7: Distribution of the top 20 most frequent *nif*H TRFs over all Sites. Sites are: Sedge Meadow (SM), Cassiope Heath (CH), Riverside Willow (RW), Upland Granite (UG), and Upland Dolomite (UD).

Subtle changes in the distribution of the most frequently present individual *nif*H TRFs explained some of the differences between sites (Figure 7). *nif*H TRFs with the greatest freq at the SM were 83 (0.874), 192 (0.846), 141 (0.840), 92 (0.838), and 122/222 (both 0.806) (Figure 7). At the CH, TRF83 (0.888) was also the most frequent, but then TRF distribution differs: 102 (0.698), 141 (0.670), 122 (0.658), and 162 (0.627). RW TRFs present with the highest frequency are 222 (0.895), 83 (0.893), 182 (0.881), 162 (0.874), 141 (0.858). Most frequent at UG is TRF 83 (0.884), followed by 92 (0.838), 192 (0.767), 141 (0.721), and 162 (0.701). The UD site is different from most with a very uniform frequency of common TRFs: 282 (0.935), 122, 182 and 322 (all 0.931), and 222 (0.928).

Figure 9 shows *nosZ* relative abundance data separating loosely by site and includes an overlay of the most abundant *nosZ* TRFs. This view of a three dimensional NMS solution explains 76.7% of the variance; axis 3 explains an additional 16.7% (cumulative r^2=0.934). Final stress was fair (10.46472) and final instability was 0.00001 after 108 iterations. Pairwise PERMANOVA comparisons confirm dissimilarity between the Sedge Meadow site and all others: SM and CH (p=0.0274), SM and RW (p=0.0304), SM and UG (p=0.0291), and SM and UD (p=0.0267). There were significant differences when the two additional lowland sites were compared individually to the upland sites and when the upland sites were compared to each other: CH and UG (p=0.0309) or UD (p=0.0255), RW and UG (p=0.0278) or UD (p=0.0301), and finally UG and UD (p=0.0310). These relationships are compared in Figure 8. Differences in the distribution of the top 20 most abundant *nosZ* TRFs over all five sites are shown in Figure 10.

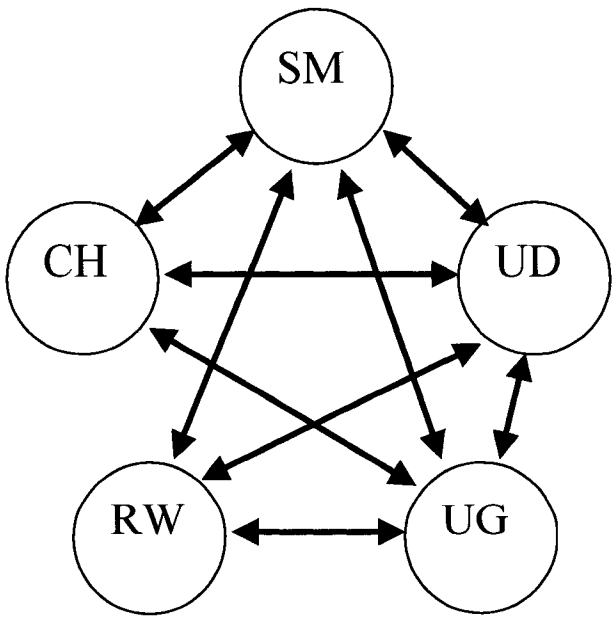

Figure 8: Arrows indicate significant differences in the relative abundance of *nosZ* TRFs between sites.

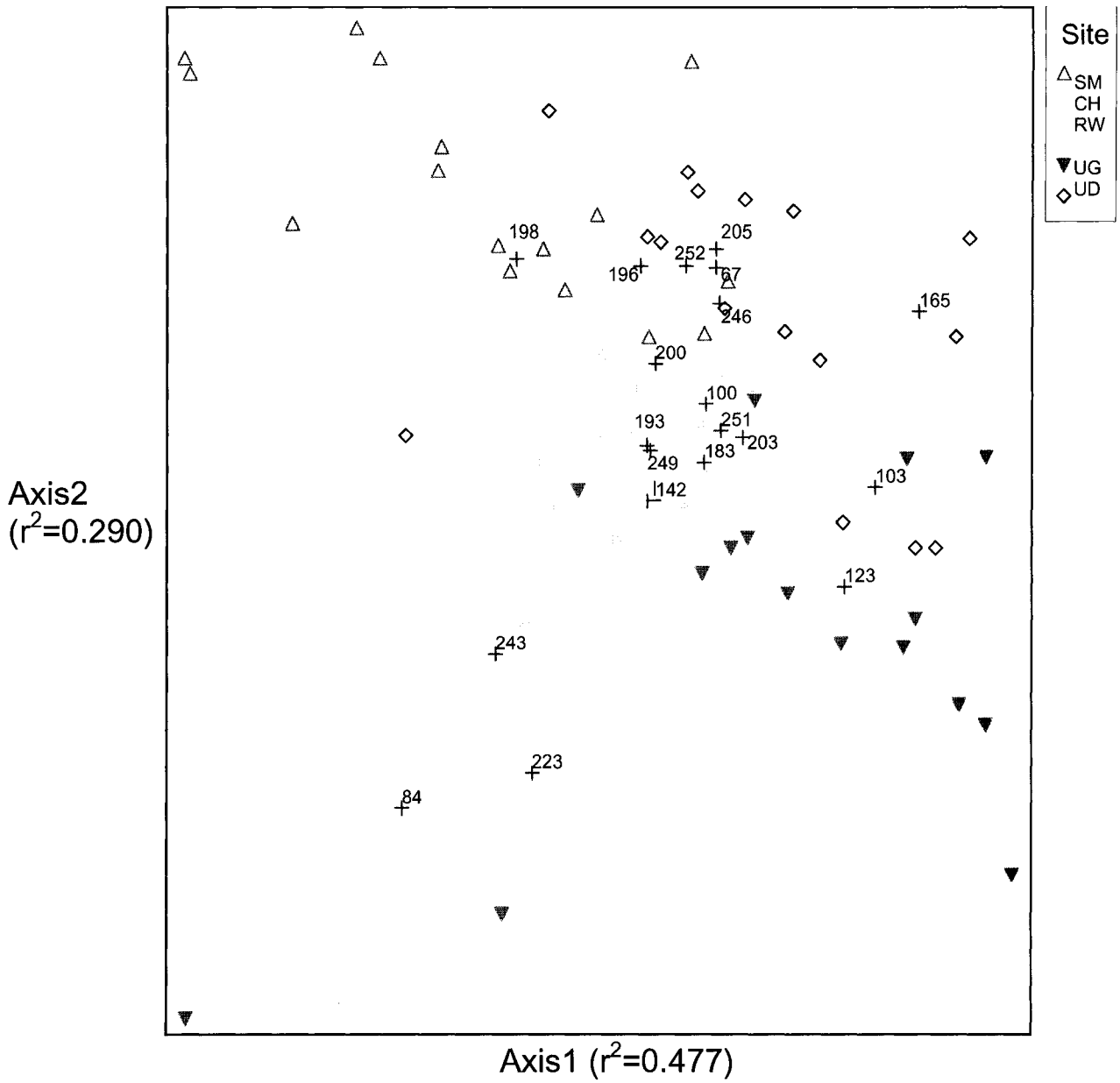

Figure 9: NMS plot of *nosZ* TRF relative abundance by Site showing distribution of the 20 most abundant TRFs p=0.0196. Sites are: Sedge Meadow (SM), Cassiope Heath (CH), Riverside Willow (RW), Upland Granite (UG), and Upland Dolomite (UD). Crosses mark TRF location and numbers reflect fragment length in base pairs (bp).

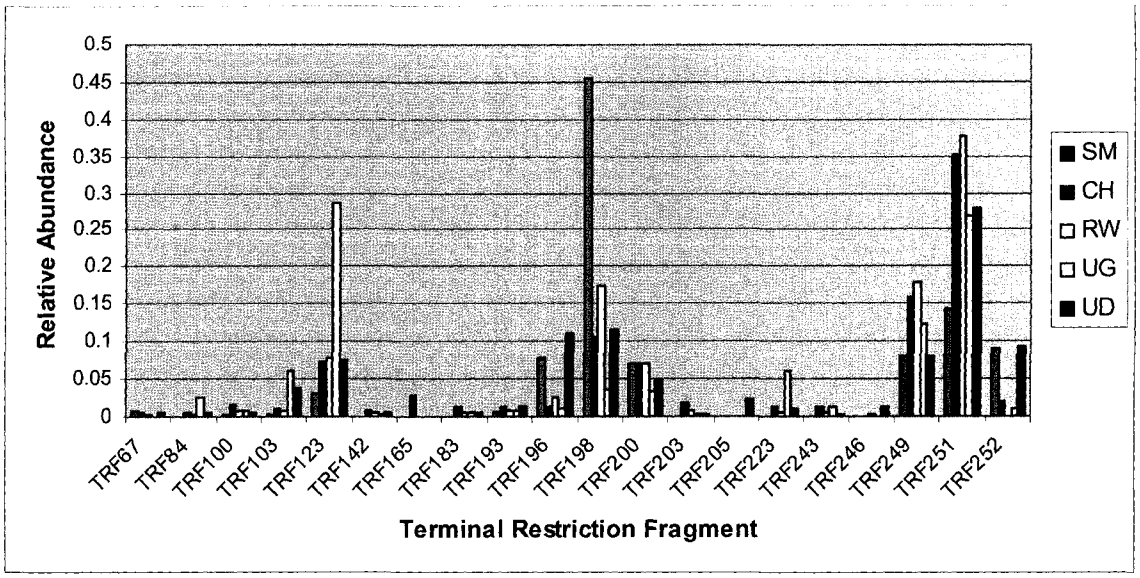

Figure 10: Distribution of the top 20 most abundant *nosZ* TRFs over all Sites

The Sedge Meadow is clearly dominated by TRF 198 (45.4%) while TRF 251 only makes up 14.4% of the gene community followed by TRF 252 (9.0%), TRF 249 (8.1%), and TRF 196 (7.8%) (Figure 10). TRF 251 is the most abundant at CH (35.1%), RW (37.7%) and UD (28.0%). The next most abundant at CH is TRF 249 (15.8%) followed by TRF198 at 10.5%, TRF 123 (7.4%), and TRF 200 (7.2%). TRFs 249 (17.8%), 198 (17.3%), 123 (7.7%), and 200 (7.0%) complete the RW community. The remainder of the UD site is completed by TRFs 198 (11.5%), 196 (11.1%), 252 (9.4%), and 249 (8.0%). At the UG site, TRF 123 is slightly more abundant than TRF 251 (28.6% vs. 26.8%). Other TRFs with substantial abundance are 249 (12.2%), 223 (6.1%), and 103 (6.0%).

Figure 12 is an NMS graph showing *nif*H relative abundance data separating clearly by site; it includes an overlay of the most abundant *nif*H TRFs across all sites. This view of a three dimensional solution explains 51.4% of the variance; axis 3 explains an additional 26.1% (cumulative r^2=0.775). Final stress was fair (14.91999) and final instability was 0.00001 after 86 iterations. Pairwise PERMANOVA comparisons confirmed dissimilarity between the Sedge Meadow site and all others: SM and CH (p=0.0314), SM and RW (p=0.0273), SM and UG (p=0.0292), and SM and UD (p=0.0283). In addition, there were significant differences between CH and RW (p=0.0298), CH and UD (p=0.0293), RW and UG (p=0.0313), and finally RW and UD (p=0.0280). These dissimilarities are illustrated more clearly in Figure 11. Figure 13 shows the top 20 most abundant *nif*H TRFs over all five sites, and how they were distributed.

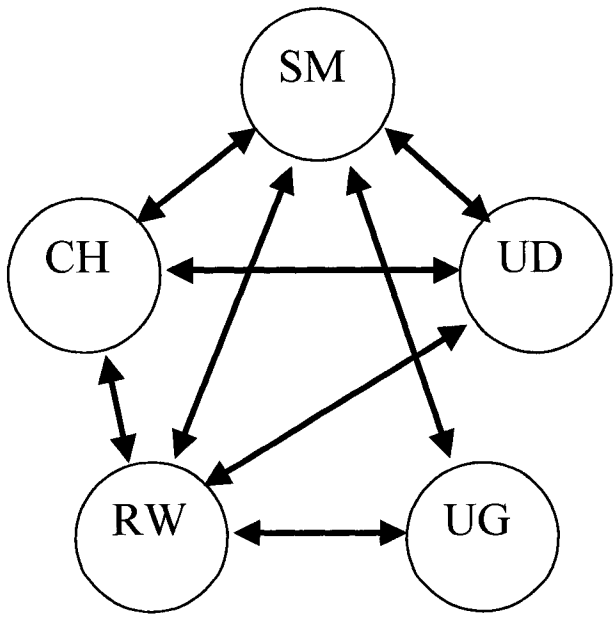

Figure 11: Arrows indicate significant differences in the relative abundance of *nif*H TRFs between sites.

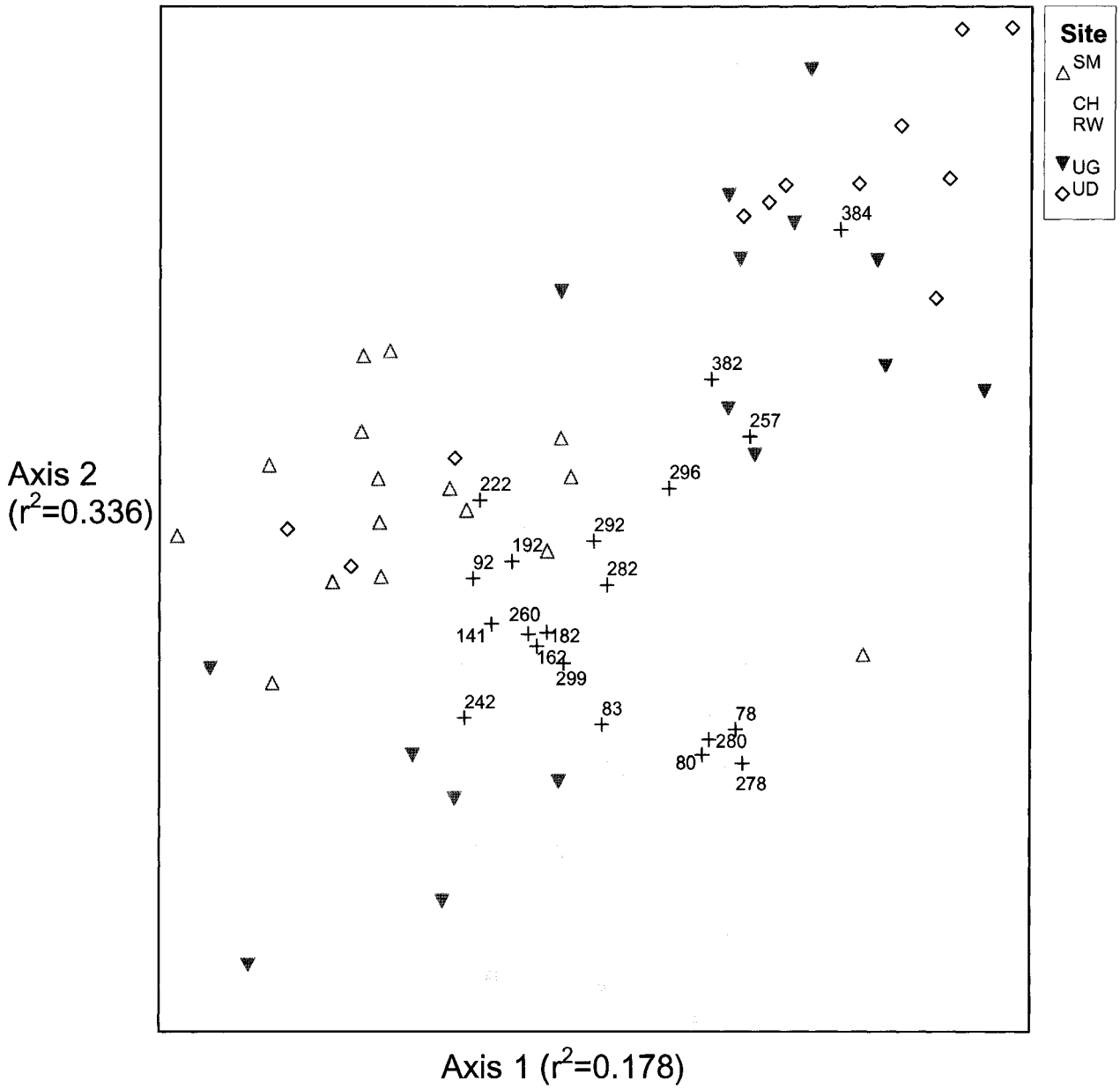

Figure 12: NMS plot of *nif*H TRF relative abundance by Site showing distribution of the 20 most abundant TRFs p=0.0196. Sites are: Sedge Meadow (SM), Cassiope Heath (CH), Riverside Willow (RW), Upland Granite (UG), and Upland Dolomite (UD). Crosses mark TRF location and numbers reflect fragment length in base pairs (bp).

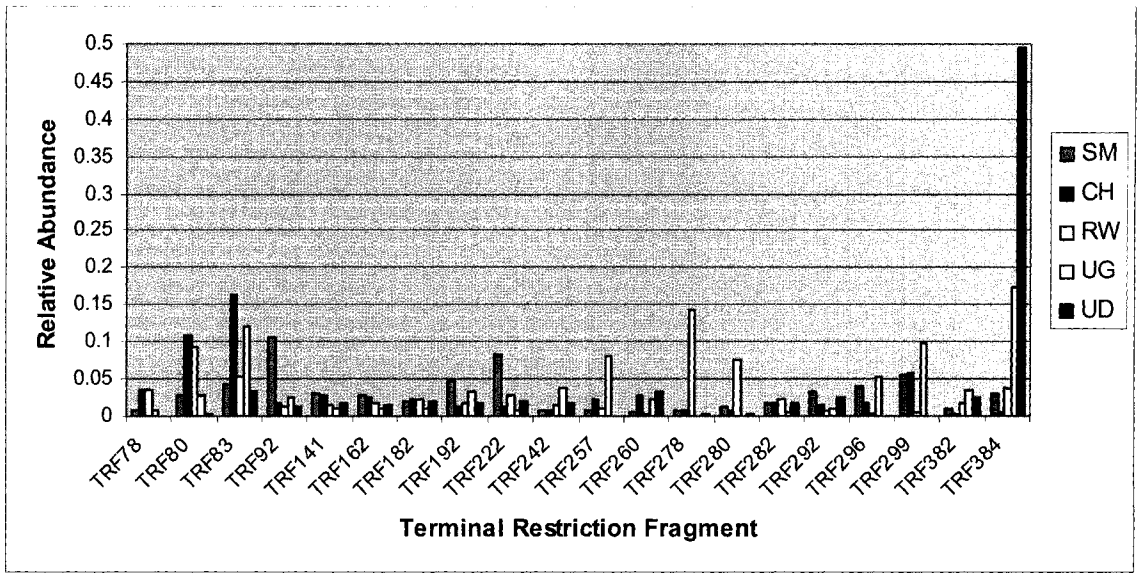

Figure 13: Distribution of the top 20 most abundant *nif*H TRFs over all sites

The relative abundance of individual *nif*H TRFs shows that each site had a unique distribution of genotypes (Figure 13). The SM site was composed of a number of different genotypes in low abundance: TRFs 92 (10.6%), 222 (8.4%), 299 (5.4%), 192 (4.7%) and 83 (4.2%). CH was structured instead by TRF 83 (16.2%), in addition to TRF 80 (10.7%), TRF 299 (5.4%), TRF 78 (3.6%), and TRF 260 (2.7%). The most abundant TRFs at RW were 278 (14.4%), 80 (9.4%), 280 (7.6%), 83 (5.2%), and 384 (3.7%). The upland sites shared the most abundant TRF, although in very different proportions to the rest of the genotype community. UG was represented by TRFs 384 (17.4%), 83 (12.1%), 299 (9.7%), 257 (8.1%), and 296 (5.3%), while at UD TRF 384 accounted for almost half of the total genotype abundance (49.6%). Only a few other TRFs were represented at UD, but in low abundance: 260 (3.4%), 83 (3.3%), 292 (2.6%), and 382 (2.5%).

Depth effect

PERMANOVA tests did not support an overall effect of the factor Depth for *nosZ* frequency data, but did suggest a significant difference in the relative abundance of *nosZ* TRFs by depth over all sites (p=0.001).

Figure 14 shows *nosZ* frequency data for the Upland Dolomite site only and highlights the dissimilarity between upper and lower samples. This two dimensional NMS solution shows how the top 20 most frequent *nosZ* TRFs were distributed by depth (cumulative r^2=0.937). A good final stress of 7.70589 and final instability of 0.00001 added confidence to this ordination which required 66 iterations. PERMANOVA supported the separation of both the control samples (p=0.0301) and the OTC samples (p=0.0285) by depth.

Figure 15 shows the top 20 most frequent *nosZ* TRFs for the Upland Dolomite site only, and how they were distributed between depths. This NMS graph combines both Control and OTC samples. TRFs with the greatest presence in upper samples were 198 (0.979), 196 (0.954), 251 (0.936), 200 (0.835), and 249 (0.792), while lower samples contained TRFs 251 (0.840), 123 (0.777), 223 (0.704), 84 (0.590), and 103 (0.565)

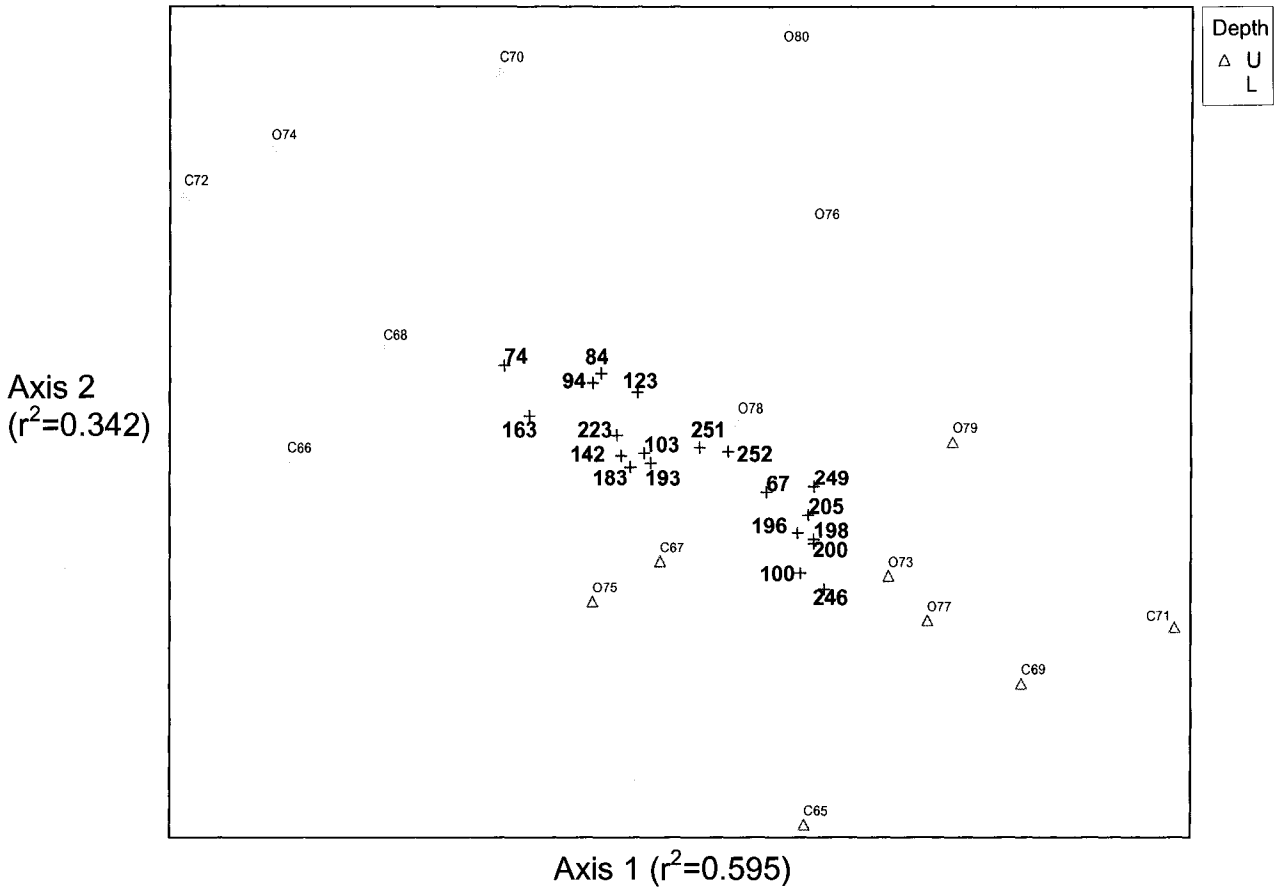

Figure 14: NMS plot of *nosZ* TRF frequency by Depth for the Upland Dolomite site showing the top 20 most frequent TRFs (p=0.0196). Depths are upper (U) or lower (L), and all numbered samples are labeled control (C) or OTC (O). Crosses mark TRF location, and bold number reflects fragment length in base pairs (bp).

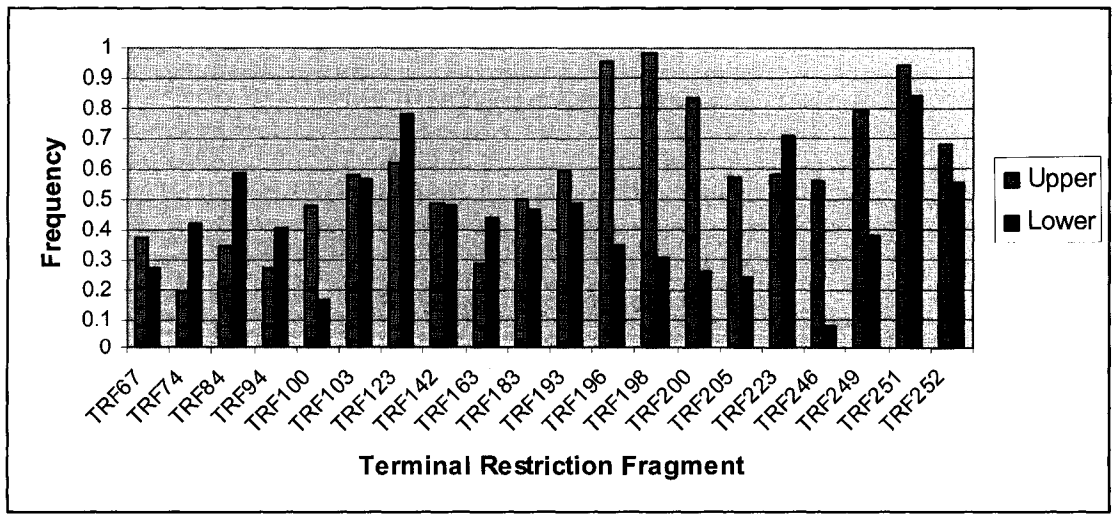

Figure 15: Distribution of the top 20 most frequent *nosZ* TRFs from Control and OTC samples of the Upland Dolomite Site

Pairwise PERMANOVA comparisons of *nosZ* relative abundance data confirmed dissimilarity between upper and lower samples at Sedge Meadow OTCs (p=0.0304). Figure 16 is the NMS ordination of Sedge Meadow samples only, highlighting those from OTCs, with Axis 1 (r^2=0.133) and Axis 2 (r^2=0.827) explaining 96.0% of the variance. This two dimensional NMS solution shows how the top 20 most abundant *nosZ* TRFs were distributed at two depths; it required 76 iterations and resulted in a good final stress of 6.99352 and final instability of 0.00001. Figure 17 shows the distribution of the top 20 most abundant *nosZ* TRFs in OTC samples from the Sedge Meadow site. Upper samples are composed of TRFs 198 (23.7%), 251 (19.7%), 196 (11.1%), 200 (9.7%), and 249 (9.4%); TRF 198 dominates lower depths (81.7%) with further contribution by TRF 252 (6.7%), TRF 196 (5.0%), TRF 251 (2.1%) and TRF 200 (1.9%).

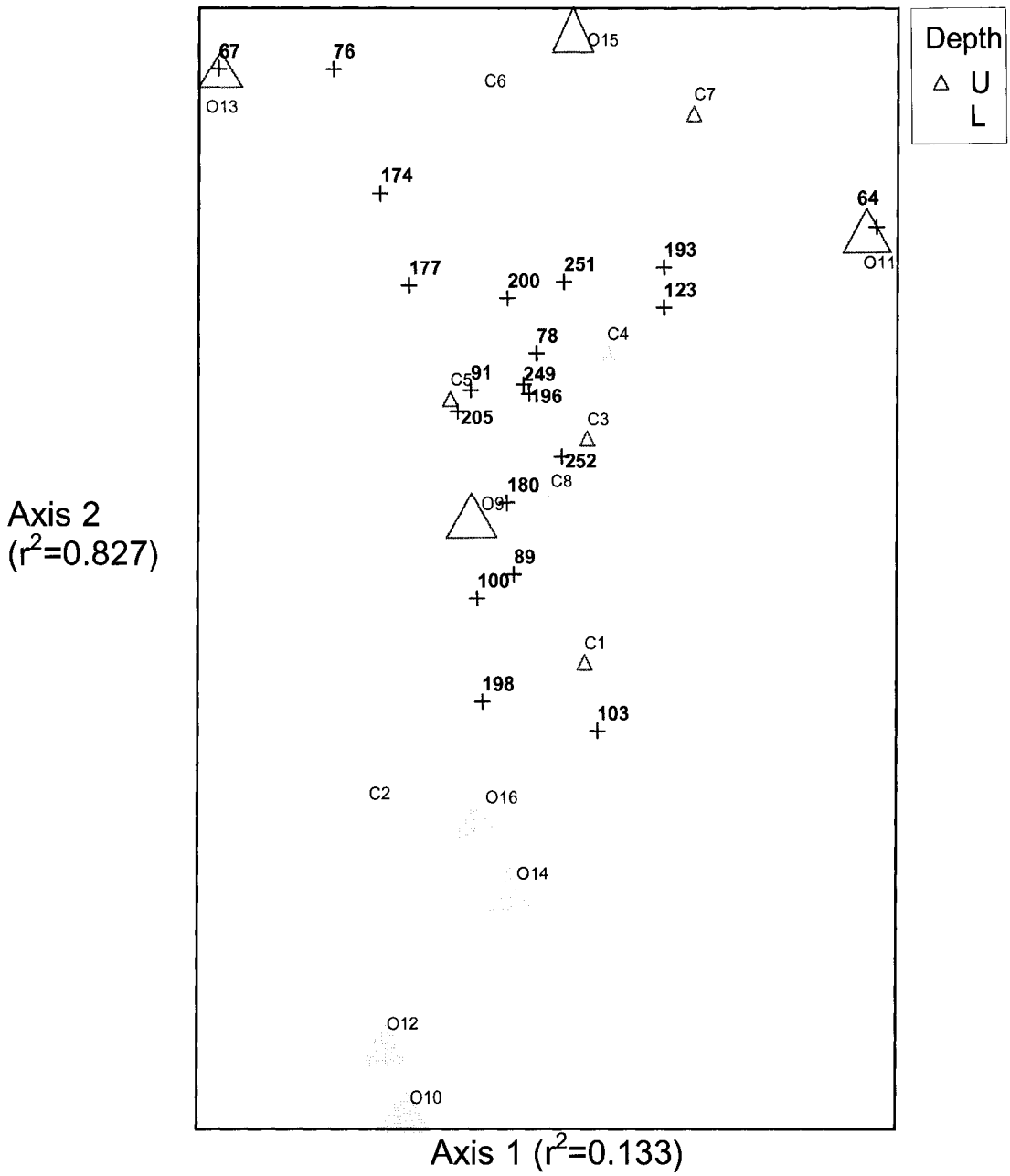

Figure 16: NMS plot of *nos*Z TRF relative abundance by Depth for the Sedge Meadow site showing the top 20 most abundant TRFs (p=0.0196). Depths are upper (U) or lower (L), and all numbered samples are labeled either control (C) or OTC (O) (OTC icons are larger in order to highlight the statistical significance of their separation). Crosses mark TRF location and numbers (bold) reflect fragment length in base pairs (bp).

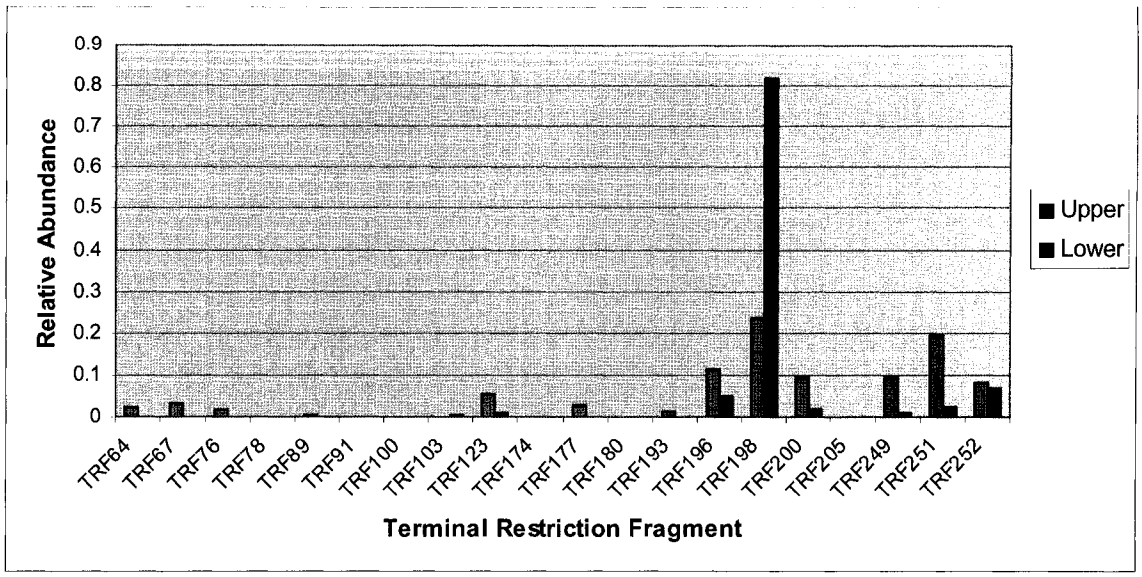

Figure 17: Distribution of the top 20 most abundant *nosZ* TRFs from OTC samples of the Sedge Meadow Site.

Pairwise PERMANOVA tests of *nosZ* relative abundance data also suggested specific differences between upper and lower samples at the Upland Dolomite control plots (p=0.0301) site. Figure 18 is a two dimensional NMS solution highlighting control samples, and showing how the top 20 most abundant *nosZ* TRFs were distributed at two depths. This solution required 52 iterations and resulted in a good final stress of 9.49054 and final instability of 0.00001. Axes 1 (r^2=0.210) and 2 (r^2=0.691) together explain 90.1% of the variance. Figure 19 shows the distribution of the top 20 most abundant *nosZ* TRFs for control samples from the Upland Dolomite Site. Upper samples were co-dominated by TRFs 196 (18.0%), 251 (17.8%), 198 (14.1%), 249 (13.7%), and 252 (7.2%), while lower samples were dominated by TRF 251 (39.2%), with TRF 252 (12.1%), TRF 123 (11.5%), TRF 103 (8.2%), and TRF 249 (6.3%) also present.

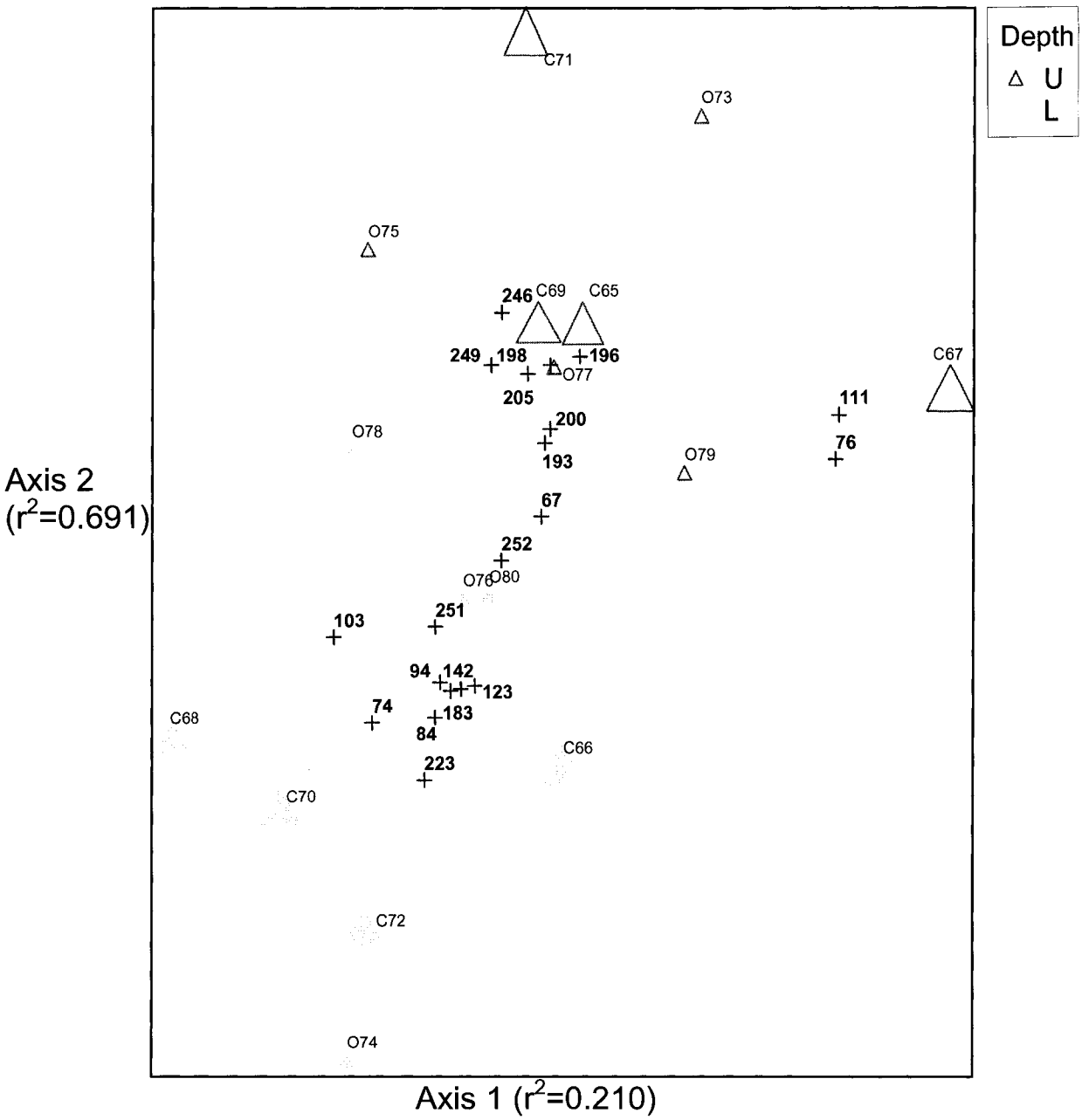

Figure 18: NMS plot of *nos*Z TRF relative abundance by Depth for the Upland Dolomite site showing the top 20 most abundant TRFs (p=0.0196). Depths are upper (U) or lower (L) and all numbered samples are labeled either control (C) or OTC (O) (control icons are larger in order to highlight the statistical significance of their separation). Crosses mark TRF location and numbers (bold) reflect fragment length in base pairs (bp).

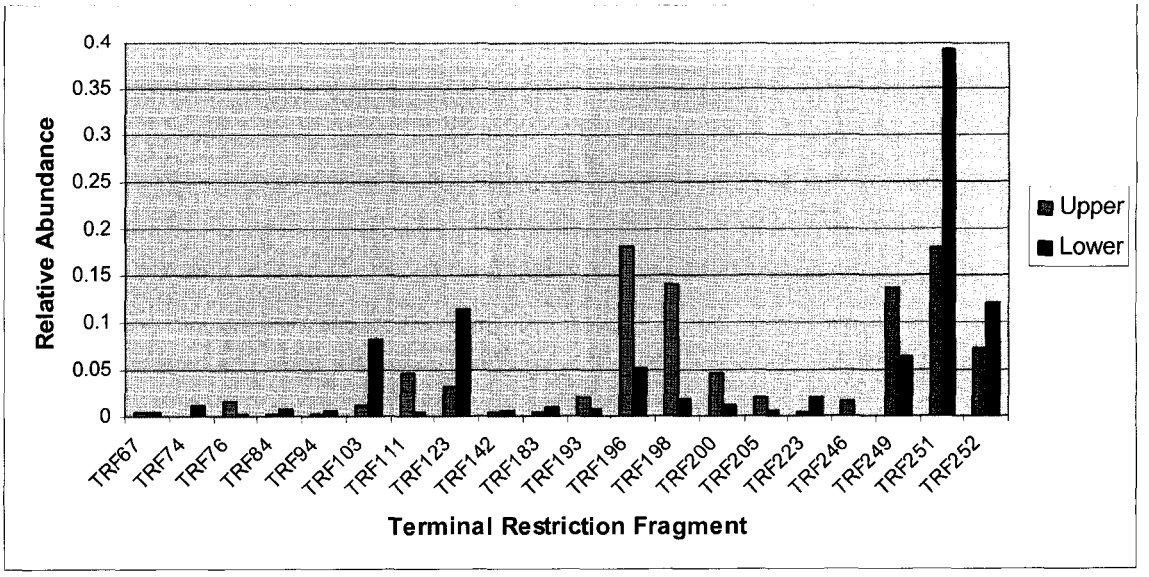

Figure 19: Distribution of the top 20 most abundant *nos*Z TRFs from Control samples of the Upland Dolomite Site

PERMANOVA tests suggested a significant difference in the frequency and relative abundance of *nif*H TRFs by depth over all sites (p=0.0001). Figure 20 is a two dimensional NMS ordination of Sedge Meadow *nif*H frequency data showing both control and OTC samples separating by depth and including the top 20 most frequent *nif*H TRFs at this site (cumulative r^2=0.938). This solution required 45 iterations and resulted in a final stress of 9.23682 and final instability of 0.00001; it reflects pairwise PERMANOVA tests showing that both control samples (p=0.0308) and OTC samples (p=0.0252) separate according to depth. Figure 21 shows the distribution of the top 20 most frequent *nif*H TRFs from both the Control and OTC samples of the Sedge Meadow Site. The upper samples had a slightly different distribution but most often included TRFs 92 (0.913), 141 and 192 (both 0.906), 182 (0.844), and 83 (0.819). Lower samples contained TRFs 83 (0.929), 222 (0.894), 122 (0.831), 192 (0.785), and 141 (0.773).

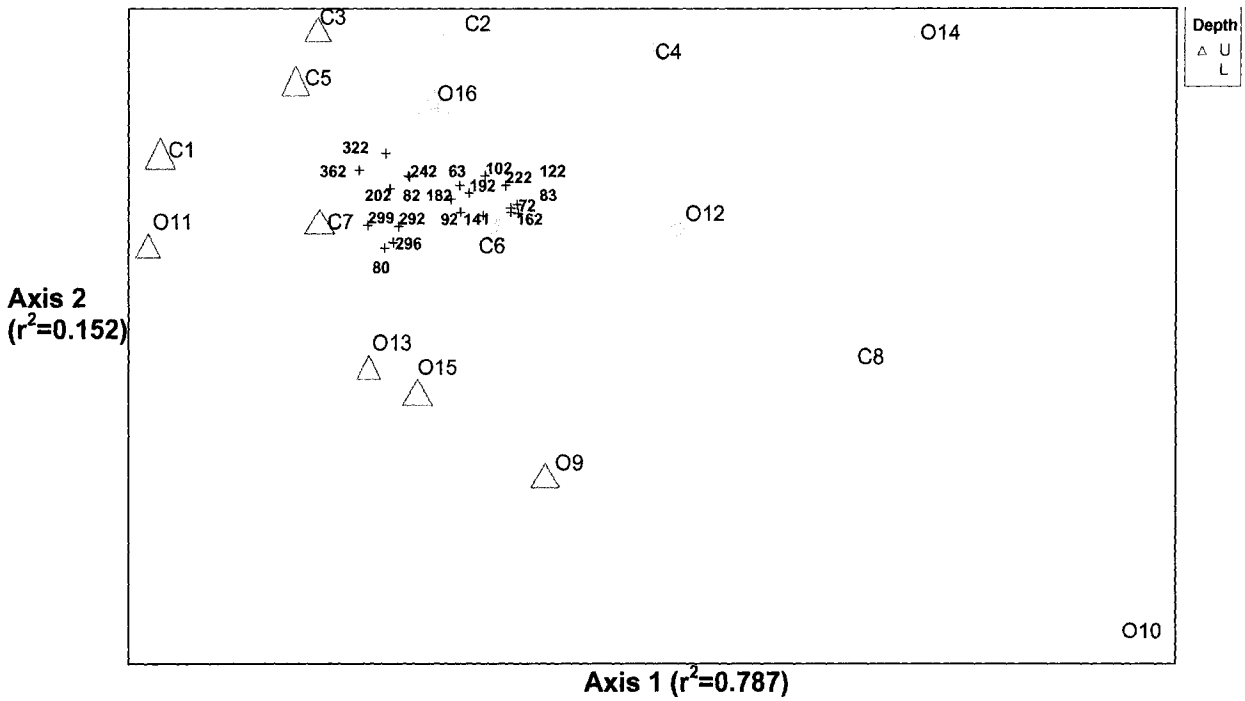

Figure 20: NMS plot of *nif*H TRF frequency by Depth for the Sedge Meadow site showing the distribution of the top 20 most frequent TRFs (p=0.0196). Depths are upper (U) or lower (L) and numbered samples are labeled either control (C) or OTC (O). Crosses mark TRF location and numbers (bold) reflect fragment length in base pairs (bp).

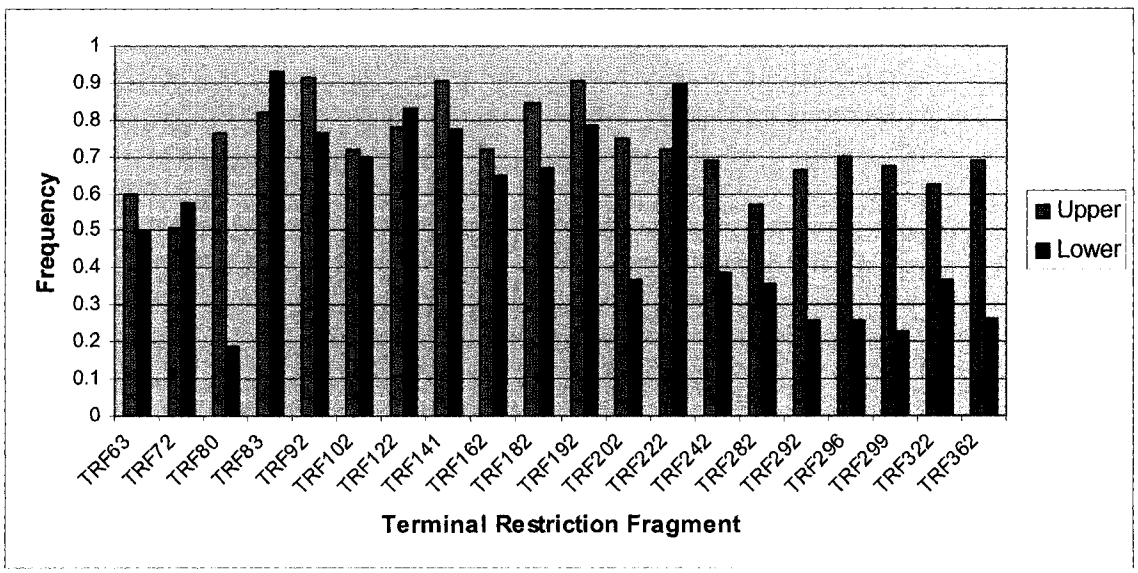

Figure 21: Distribution of the top 20 most frequent *nif*H TRFs from Control and OTC samples of the Sedge Meadow Site

Figure 22 is a two dimensional NMS ordination of Upland Granite *nif*H frequency data showing control samples separating by depth and including the top 20 most frequent *nif*H TRFs at this site (cumulative r^2=0.932). This solution required 72 iterations and resulted in a good final stress of 8.31342 and final instability of 0.00001. PERMANOVA supported the separation of control samples by depth (p=0.0319). Figure 23 represents the top 20 most frequent *nif*H TRFs from the Upland Granite Site; only the control samples are shown in this graph. The upper samples were represented by TRFs 384 (0.846), 83 (0.788), 257 (0.713), 80 (0.692), and 92 (0.642), while lower samples contained a high frequency of TRF 299 and TRF 83 (both 1.0), plus TRF 80 (0.917), TRF 92 (0.833), and TRF 192 (0.750).

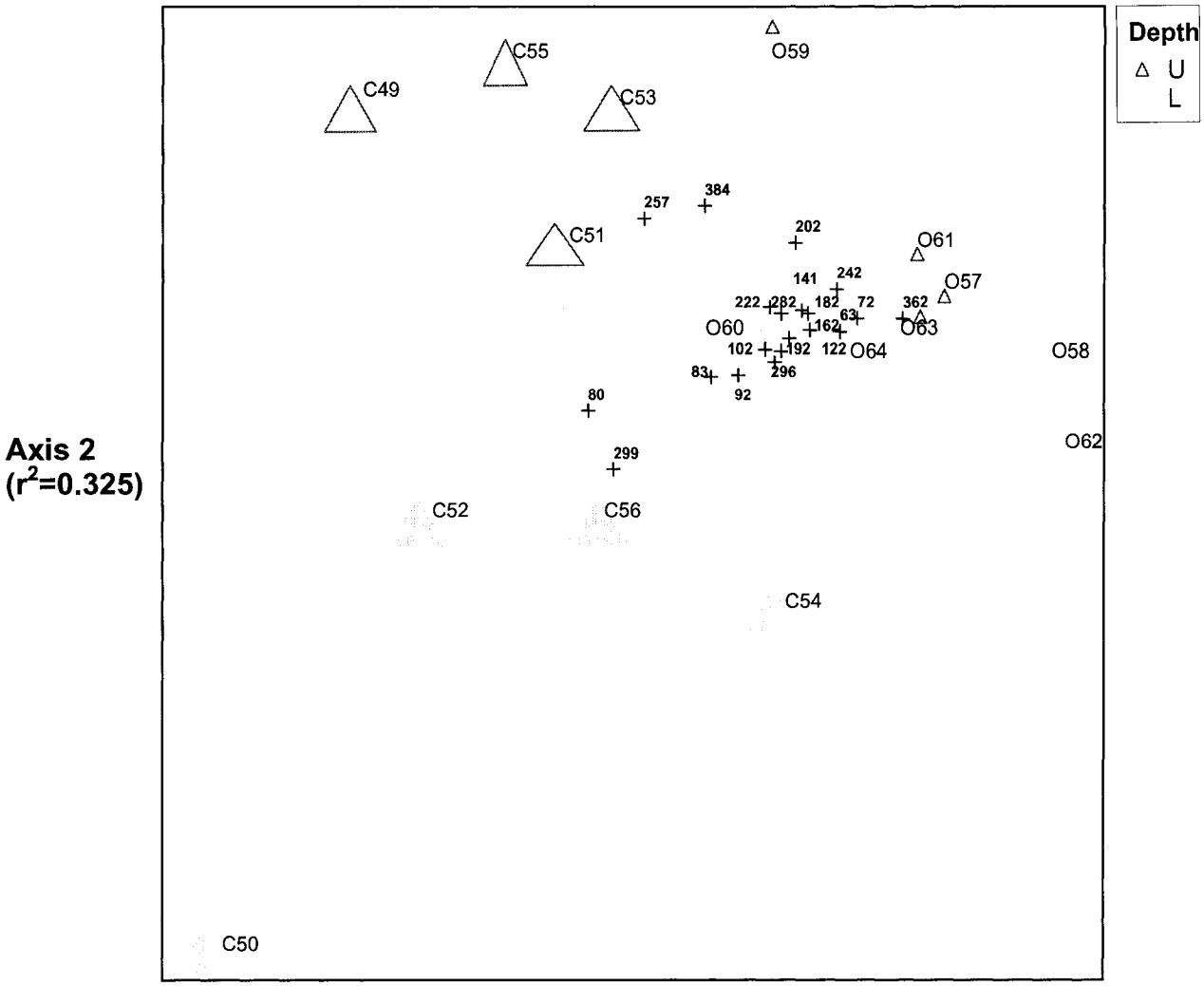

Figure 22: NMS plot of *nif*H TRF frequency by Depth for the Upland Granite site showing the top 20 most frequent TRFs (p=0.0196). Depths are upper (U) or lower (L) and numbered samples are labeled control (C) or OTC (O) (control icons are larger in order to highlight the statistical significance of their separation). Crosses mark TRF location and numbers (bold) reflect fragment length in base pairs (bp).

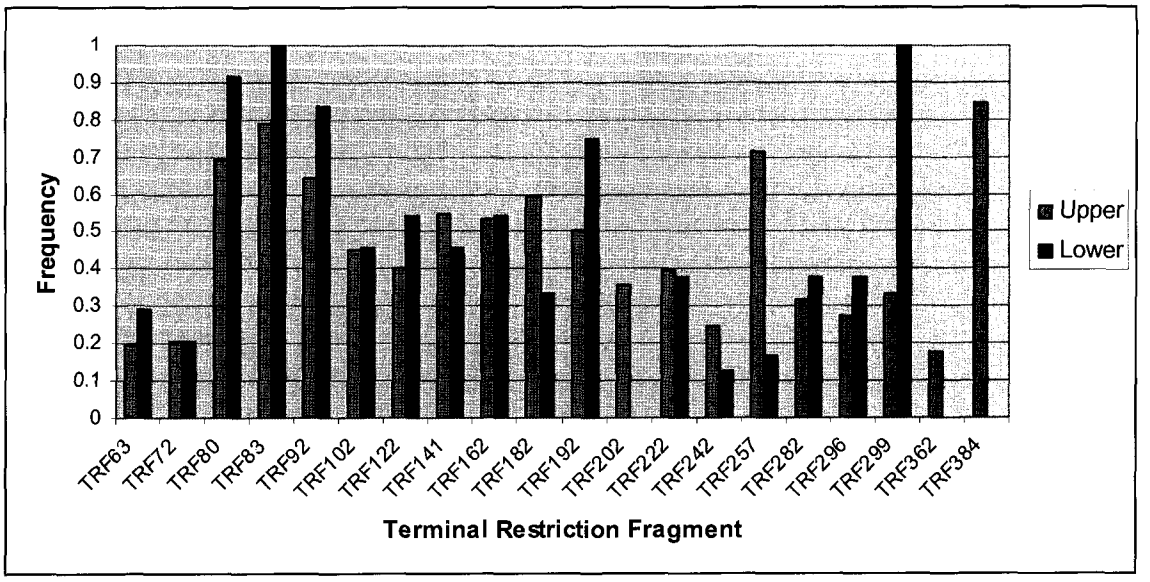

Figure 23: Distribution of the top 20 most frequent *nif*H TRFs from Control samples of the Upland Granite Site

Pairwise PERMANOVA tests of *nif*H relative abundance data confirmed dissimilarity between upper and lower samples at Sedge Meadow control plots (p=0.0314) and Sedge Meadow OTCs (p=0.0273). Figure 24 is the NMS ordination of Sedge Meadow samples only with Axis 1 (r^2=0.218) and Axis 2 (r^2=0.618) explaining 83.6% of the variance. This two dimensional solution shows control and OTC samples plus the top 20 most abundant *nif*H TRFs at this site; it required 59 iterations and resulted in a final stress of 12.19391 and final instability of 0.00000. Figure 25 shows the distribution of the top 20 most abundant *nif*H TRFs from both the Control and OTC samples of the Sedge Meadow Site. Upper samples were structured by TRFs 92 (13.3%), 299 (7.0%), 296 (6.6%), 292 (5.6%), and 80 (4.6%), while lower samples were dominated by TRFs 222 (15.1%), 92 (8.0%), 192 (6.3%), 83 (5.8%), and 162 (4.6%).

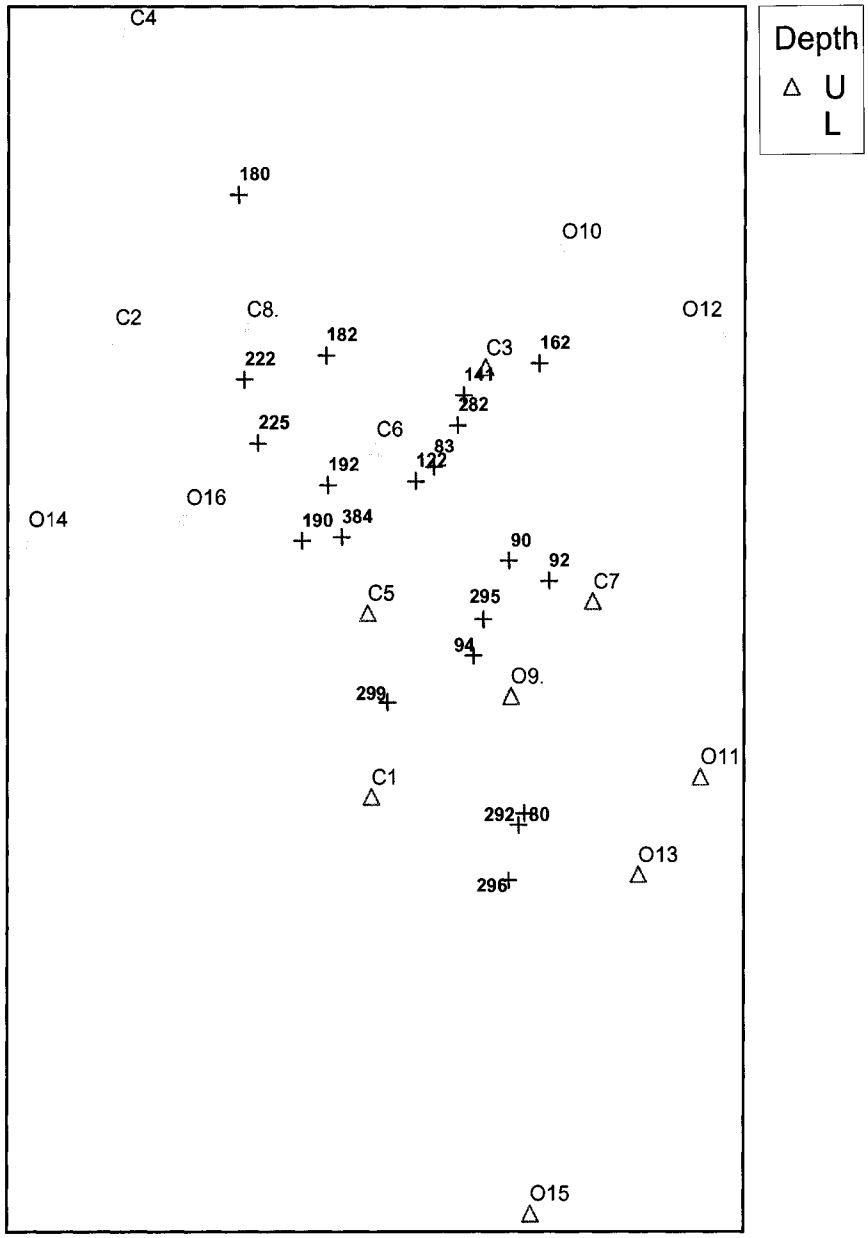

Figure 24: NMS plot of *nif*H TRF relative abundance by Depth for the Sedge Meadow site showing the distribution of the top 20 most abundant TRFs (p=0.0196). Depths are upper (U) or lower (L) and all numbered samples are labeled control (C) or OTC (O). Crosses mark TRF location and numbers (bold) reflect fragment length in base pairs (bp).

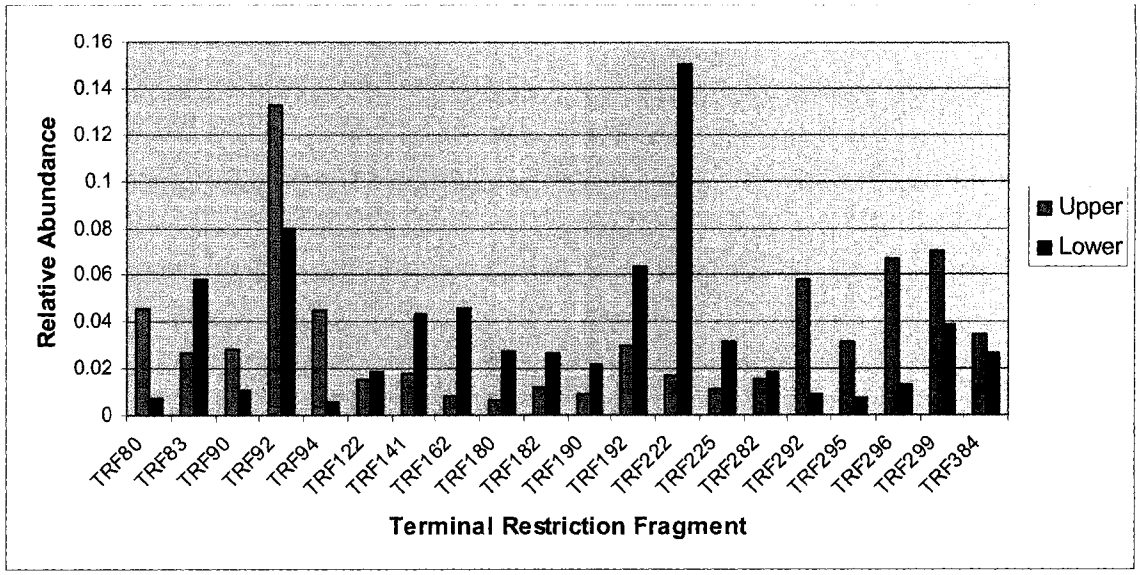

Figure 25: Distribution of the top 20 most abundant *nif*H TRFs from Control and OTC samples of the Sedge Meadow Site.

Pairwise PERMANOVA tests also suggested specific differences between control plot samples at both the Upland Granite (p=0.0293) and Upland Dolomite (p=0.0280) sites. Figure 26 is the NMS ordination of the UG site identifying control samples and the top 20 most abundant *nif*H TRFs at this site. This two dimensional solution required 103 iterations and resulted in a final stress of 13.70635 and final instability of 0.00000. Axes 1 (r^2=0.335) and 2 (r^2=0.520) plus Axes 2 together explain 85.4% of the variance. A reliable NMS image of the UD site was not generated, possibly due to the limited (and sometimes absent) lower samples. Figure 27 represents the top 20 most abundant *nif*H TRFs from the Upland Granite Site; only the control samples are shown in this graph. The most abundant TRF in upper samples, not present in lower, was TRF 384 (36.9%), followed by TRF 257 (17.1%), TRF 83 (9.8%), TRF 382 (5.7%), and TRF 254 (4.3%). Lower samples were represented by TRFs 299 (28.6%), 83 (27.2%), 260(7.1%), 92 (6.1%), and 192 (5.6%).

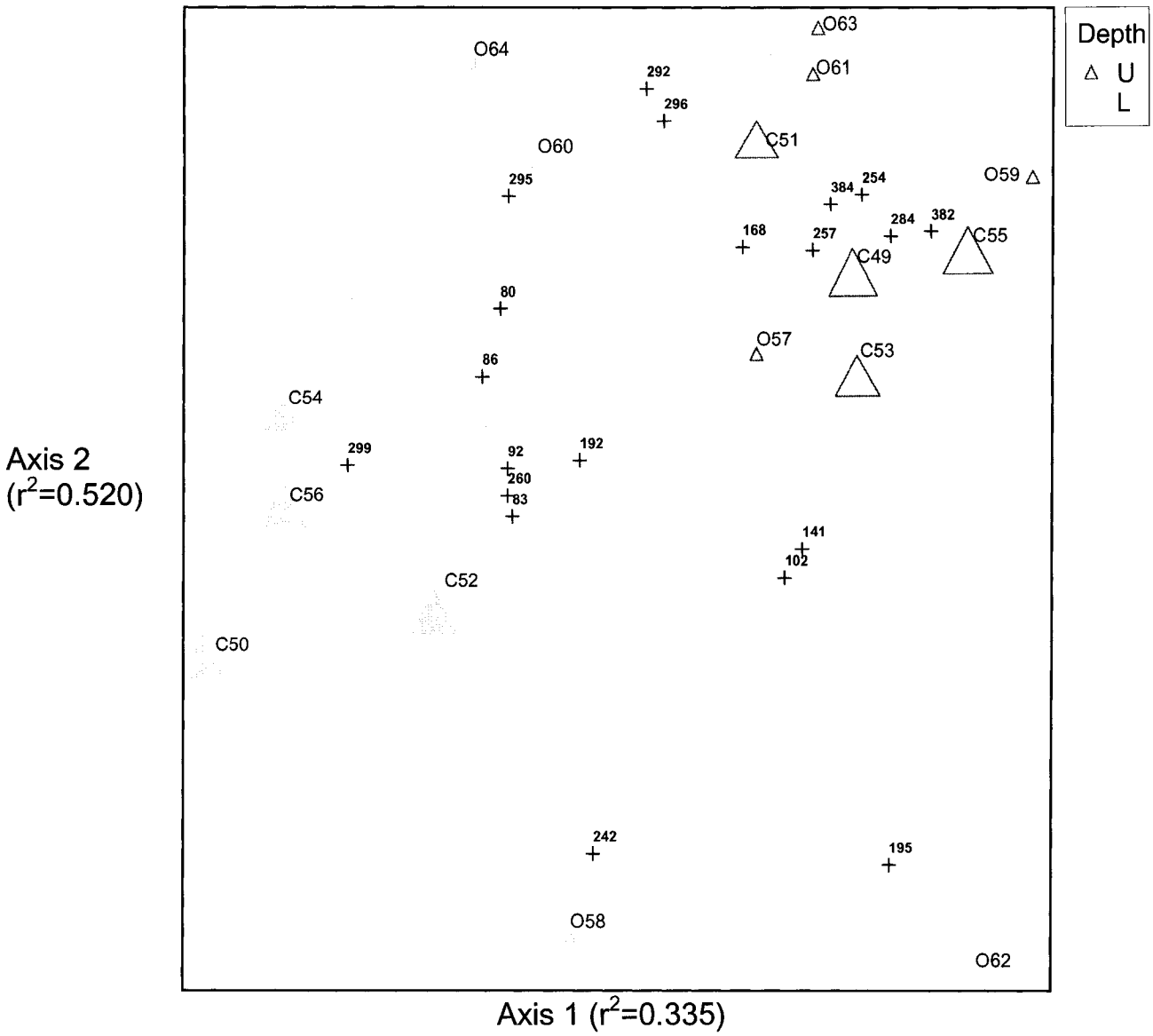

Figure 26: NMS plot of *nif*H TRF relative abundance by Depth for the Upland Granite site showing the top 20 most abundant TRFs (p=0.0392). Depths are upper (U) or lower (L) and all numbered samples are labeled Control (C) or OTC (O) (control icons are larger in order to highlight the statistical significance of their separation). Crosses mark TRF location and numbers (bold) reflect fragment length in base pairs (bp).

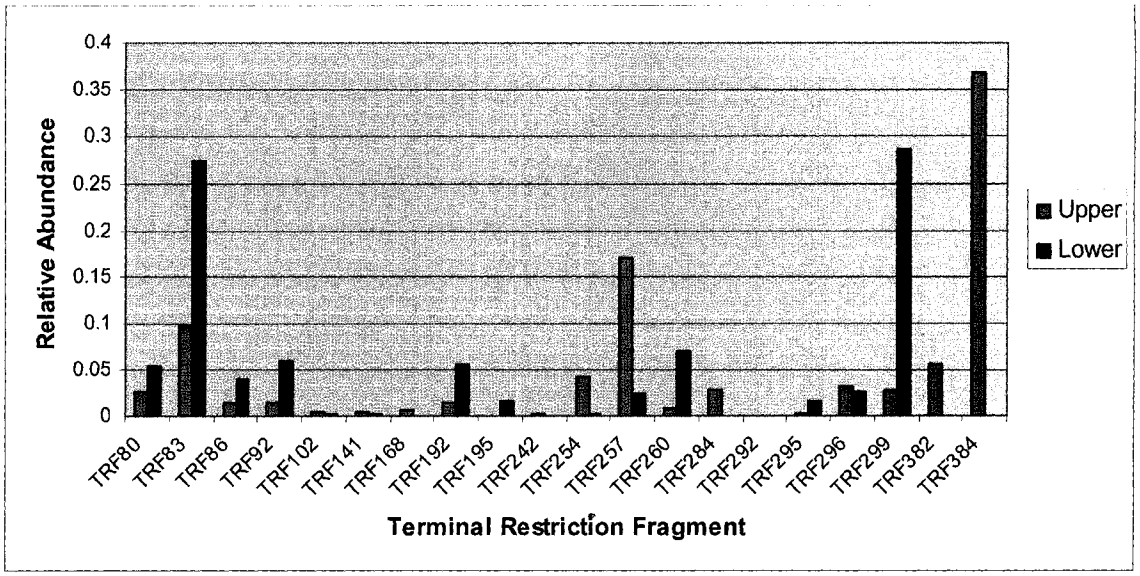

Figure 27: Distribution of the top 20 most abundant *nif*H TRFs from Control samples of the Upland Granite Site

Treatment effect

NMS ordinations did not reveal any separation of samples due to treatment effect at any site for either *nos*Z or *nif*H frequency or relative abundance data. Similarly, significance was not detected by any PERMANOVA tests for the factor treatment overall or at individual sites.

Figure 28 is a three dimensional NMS plot of *nos*Z samples from all sites showing that relative abundance data did not separate by treatment. Axis 1 and 3 (shown) explain 75.8% of the variance while axis 2 contributes 17.6% (cumulative r^2=0.934). This ordination required 126 iterations and resulted in a good final stress of 10.46479 and final instability of 0.00001.

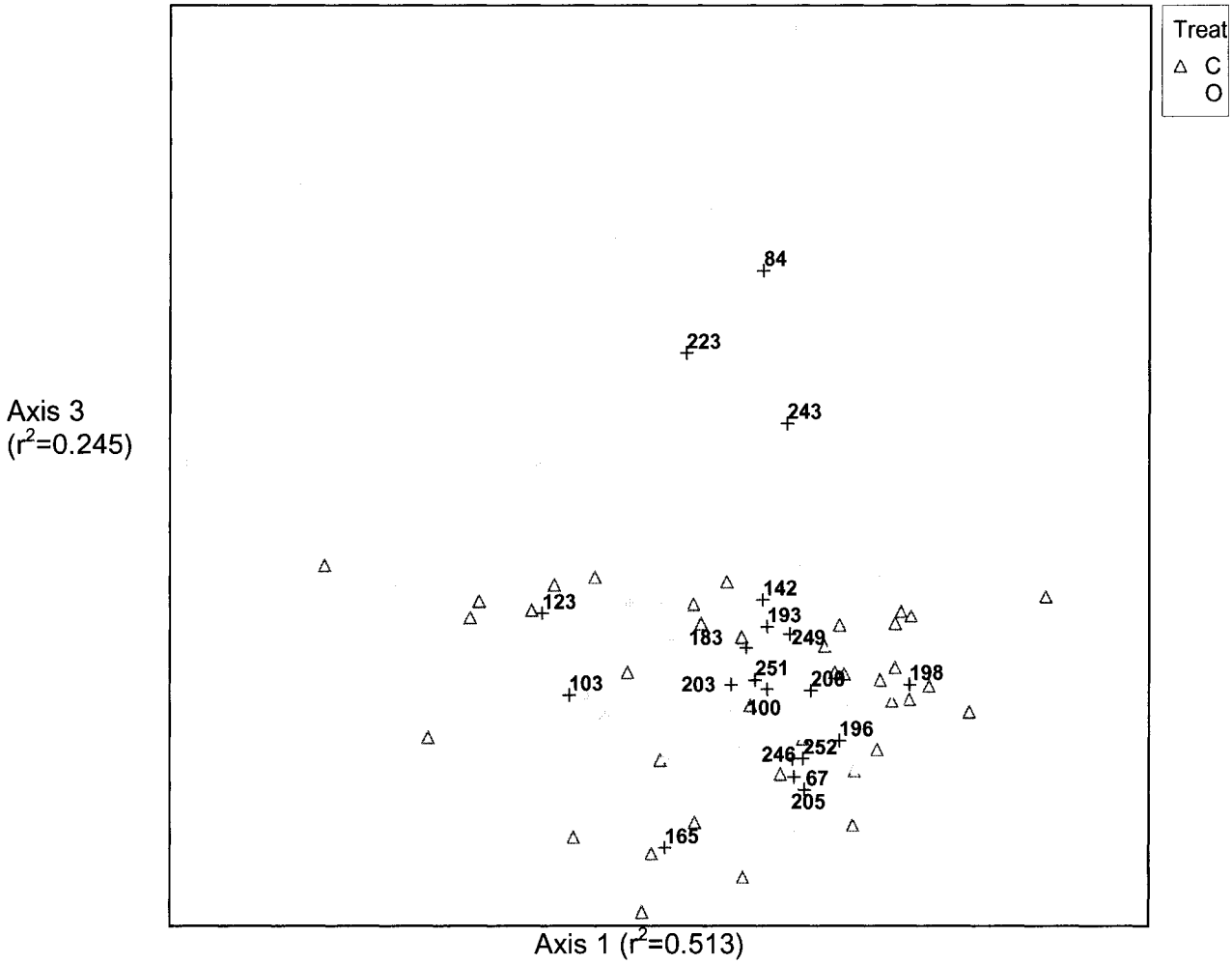

Figure 28: NMS plot of *nosZ* TRF relative abundance for all sites showing that samples do not separate by treatment (p=0.0196). Treatments are control (C) or OTC (O), and crosses mark the top 20 TRFs over all sites. Numbers reflect fragment length in base pairs (bp).

Figure 29 is a three dimensional NMS plot of *nifH* samples from all sites showing that relative abundance data did not separate by treatment. Axis 2 and 3 (shown) explain 46.8% of the variance while axis 1 contributes 29.7% (cumulative r^2=0.775). This ordination required 126 iterations and resulted in a fair final stress of 14.92058 and final instability of 0.00001.

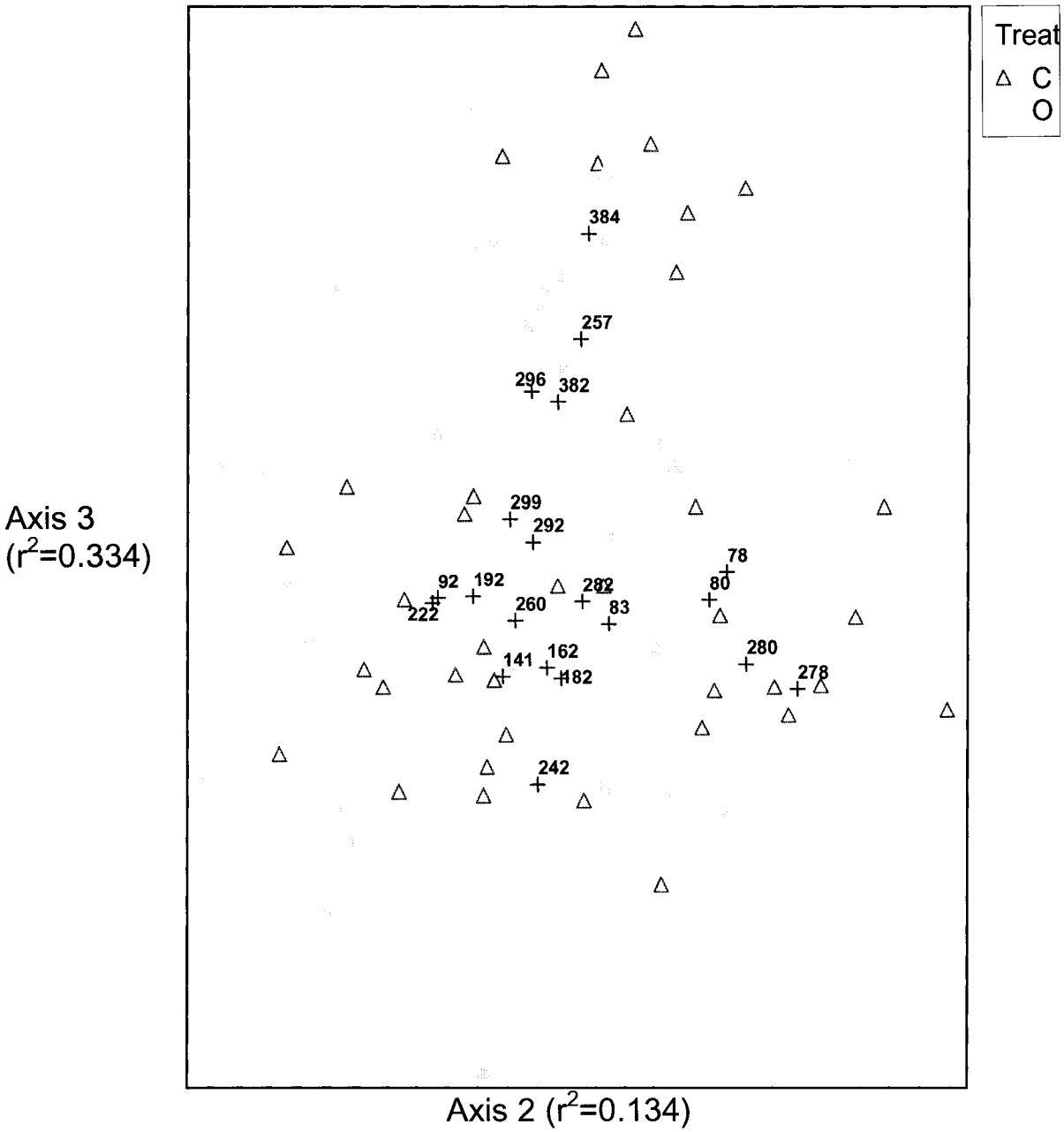

Figure 29: NMS plot of *nif*H TRF relative abundance for all sites showing that samples do not separate by treatment (p=0.0196). Treatments are control (C) or OTC (O), and crosses mark the top 20 TRFs over all sites. Numbers reflect fragment length in base pairs (bp).

2.3.2 Genotype richness

nosZ

Significant differences in the number of *nosZ* genotypes were detected overall between sites by ANOVA tests (Figure 30). Pairwise analysis showed that the Sedge Meadow (6.2 TRFs) had significantly fewer genotypes than the Cassiope Heath (18.8, $p<0.001$), Riverside Willow (16.5, $p<0.001$), and Upland Dolomite (14.5, $p<0.001$) sites. The Upland Granite site had similarly low genotype richness (5.8 TRFs). CH and RW had a similar number of unique genotypes; the CH site had significantly more genotypes than both the UG ($p<0.001$) and the UD ($p<0.001$) sites while RW differed significantly from UG ($p<0.001$). Additionally, the Upland sites differed greatly from each other with the UD displaying a far greater number of genotypes ($p<0.001$).

Nested ANOVA, in addition to showing overall site differences, described significant overall depth (Table 2) and treatment (Table 3) effects.

Table 2: Nested ANOVA showing overall significant differences in number of *nosZ* TRFs between sites ($p<0.000001$) and between depths across all sites ($p=0.018$)

	SS	DF	MS	F	p
Intercept	56942.58	1	56942.58	1365.430	0.000000
Site	10197.61	4	2549.40	61.132	0.000000
Depth(Site)	575.80	5	115.16	2.761	0.018286
Error	15388.42	369	41.70		

Table 3: Nested ANOVA showing overall significant differences in number of *nosZ* TRFs between sites ($p<0.000001$) and between treatment across all sites ($p=0.039$)

	SS	DF	MS	F	p
Intercept	56248.43	1	56248.43	1341.981	0.000000
Site	10423.34	4	2605.84	62.170	0.000000
Treat(Site)	497.78	5	99.56	2.375	0.038587
Error	15466.45	369	41.91		

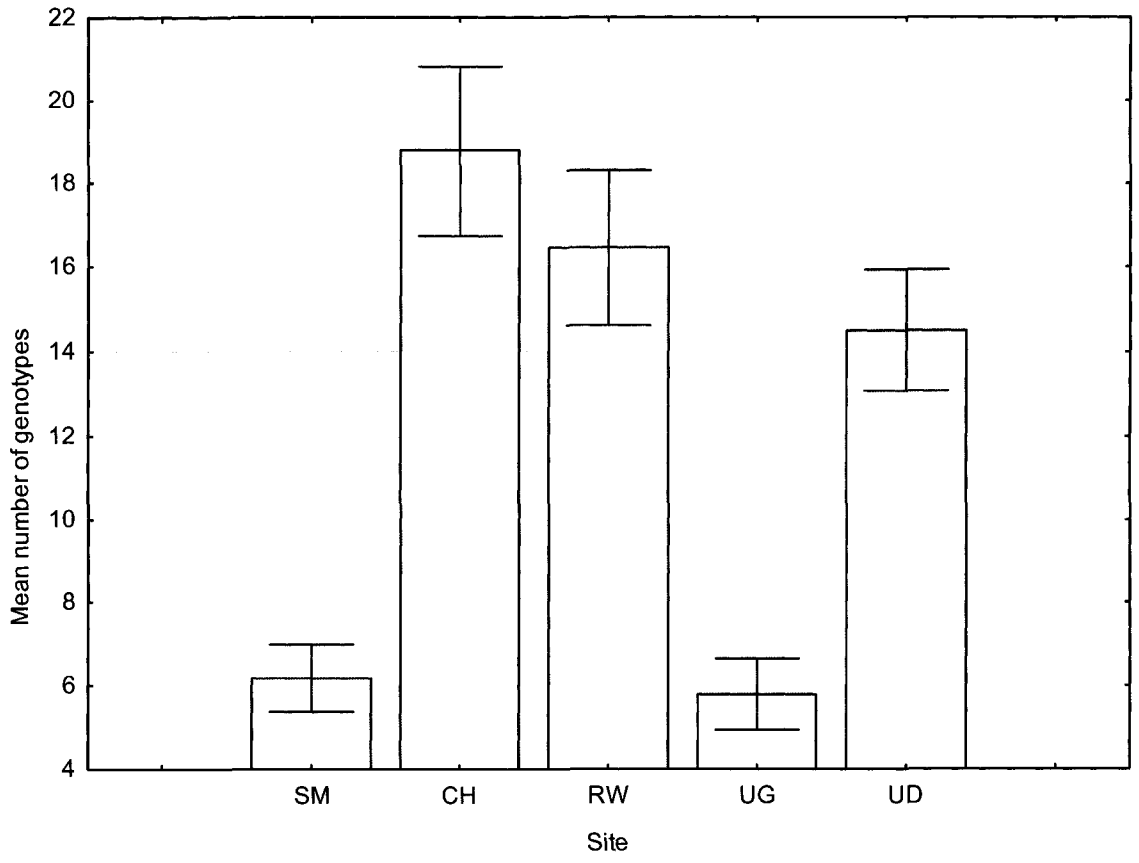

Fig 30: Mean number of *nosZ* genotypes (TRFs) at each site ($p<0.001$, confidence interval = 0.95).

Significant differences were detected overall in the number of *nosZ* genotypes between upper and lower samples (Figure 31). With the exception of the UG site, there was greater genotype richness in surface soils. One-way ANOVA confirmed differences in genotype number at SM (7.3 vs. 5.2, p=0.0078) and UD (16.8 vs. 12.4, p=0.0019).

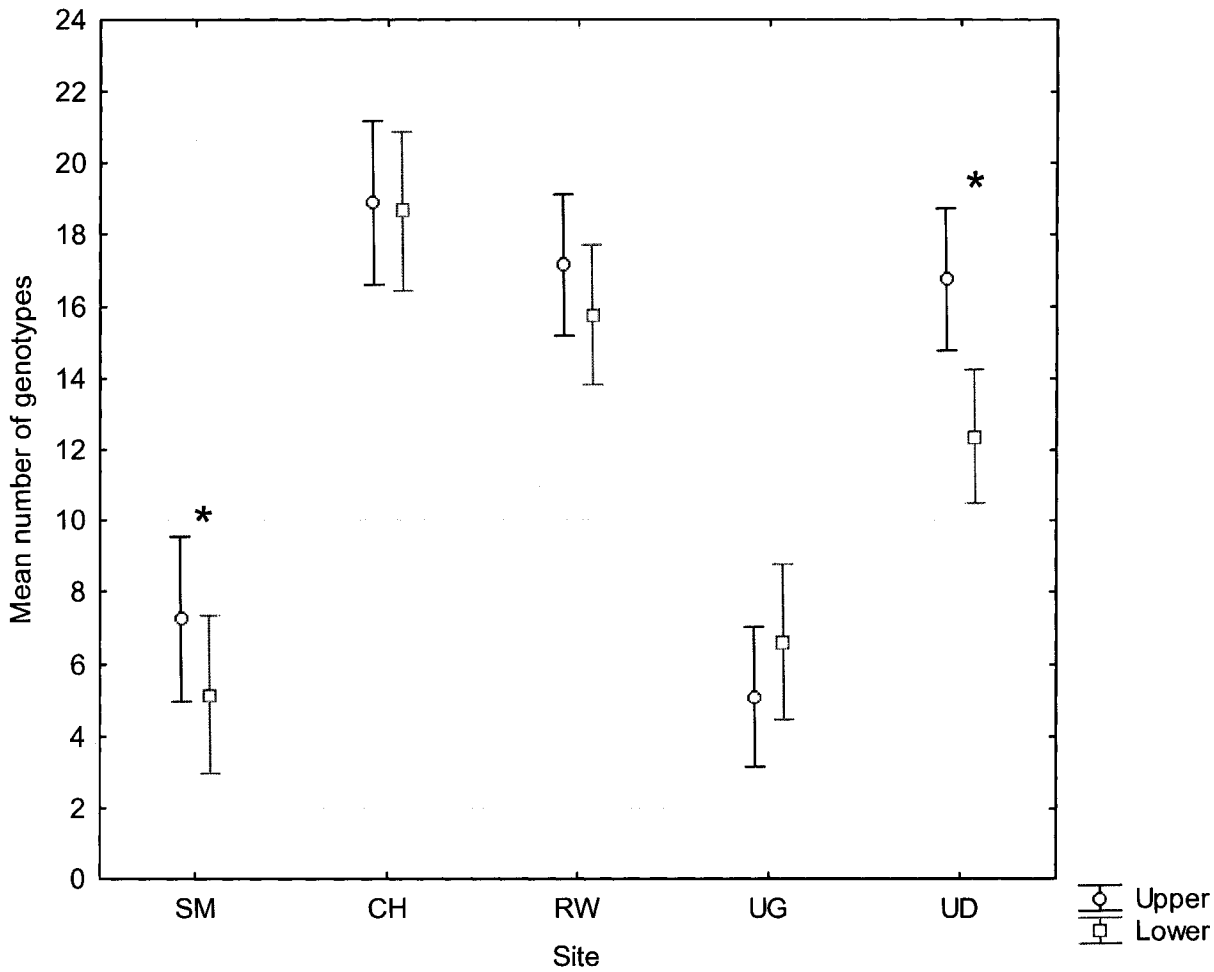

Fig 31: The mean number of *nosZ* genotypes (TRFs) differed over all sites by depth (p=0.018, CI=0.95). Significant differences at individual sites are marked with an asterisk (p<0.01).

Significant differences were detected in the number of *nosZ* genotypes between control and OTC plots (Figure 32). In general, there were fewer genotypes in OTC treatment samples. One-way ANOVA confirmed differences in genotype number at SM (7.1 vs. 5.2, p=0.018), UG (6.7 vs. 4.7, p=0.016) and UD (16.1 vs. 12.9, p=0.023).

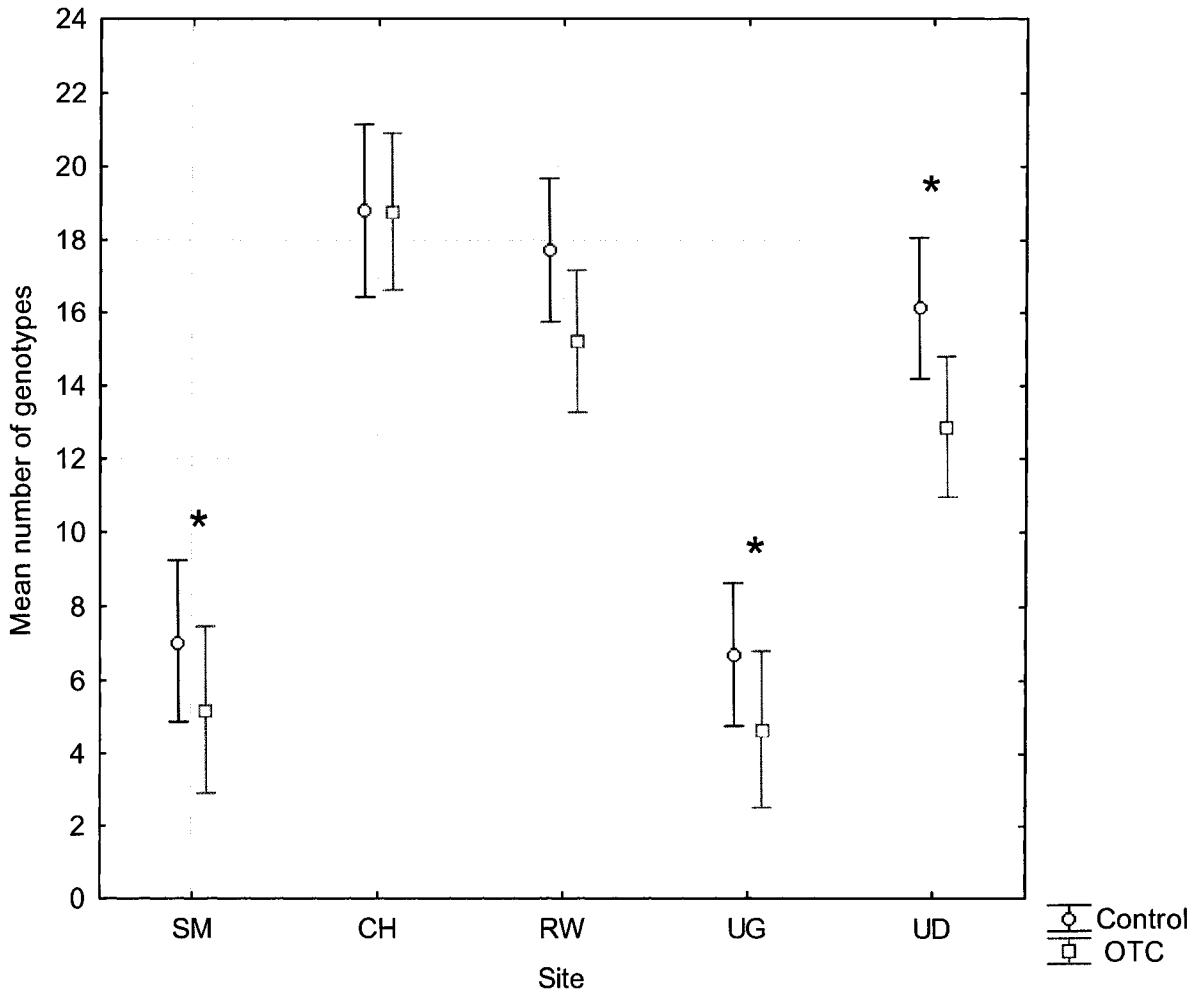

Fig 32: The mean number of *nosZ* genotypes (TRFs) differed by treatment over all sites (p=0.039, CI=0.95). Significant differences at individual sites are marked with an asterisk (p<0.05).

nifH

Significant differences in the number of *nifH* genotypes were detected overall between sites with ANOVA tests (Figure 33). Pairwise investigation showed that

richness was much lower at the CH site (15.2) versus SM (21.6, p=0.0001), RW (23.1, p<0.0001), and UD (21.4, p=0.0001), while the UG site displayed intermediate richness relative to the others (18.0). Mean number of genotypes at both upland sites were significantly lower than those at the RW (vs. UG, p=0.0001 and vs. UD, p=0.0068).

Nested ANOVA, in addition to showing overall site differences, described significant overall depth (Table 4) and treatment (Table 5) effects.

Table 4: Nested ANOVA showing overall significant differences in number of *nif*H TRFs between sites (p<0.000001) and between depths across all sites (p=0.000001)

	SS	DF	MS	F	p
Intercept	73795.51	1	73795.51	1152.939	0.000000
Site	2928.94	4	732.23	11.440	0.000000
Depth(Site)	2444.07	5	488.81	7.637	0.000001
Error	18881.89	295	64.01		

Table 5: Nested ANOVA showing overall significant differences in number of *nif*H TRFs between sites (p<0.000001) and between treatment across all sites (p<0.000001)

	SS	DF	MS	F	p
Intercept	111240.5	1	111240.5	1755.050	0.000000
Site	2640.1	4	660.0	10.413	0.000000
Treat(Site)	2627.9	5	525.6	8.292	0.000000
Error	18698.0	295	63.5		

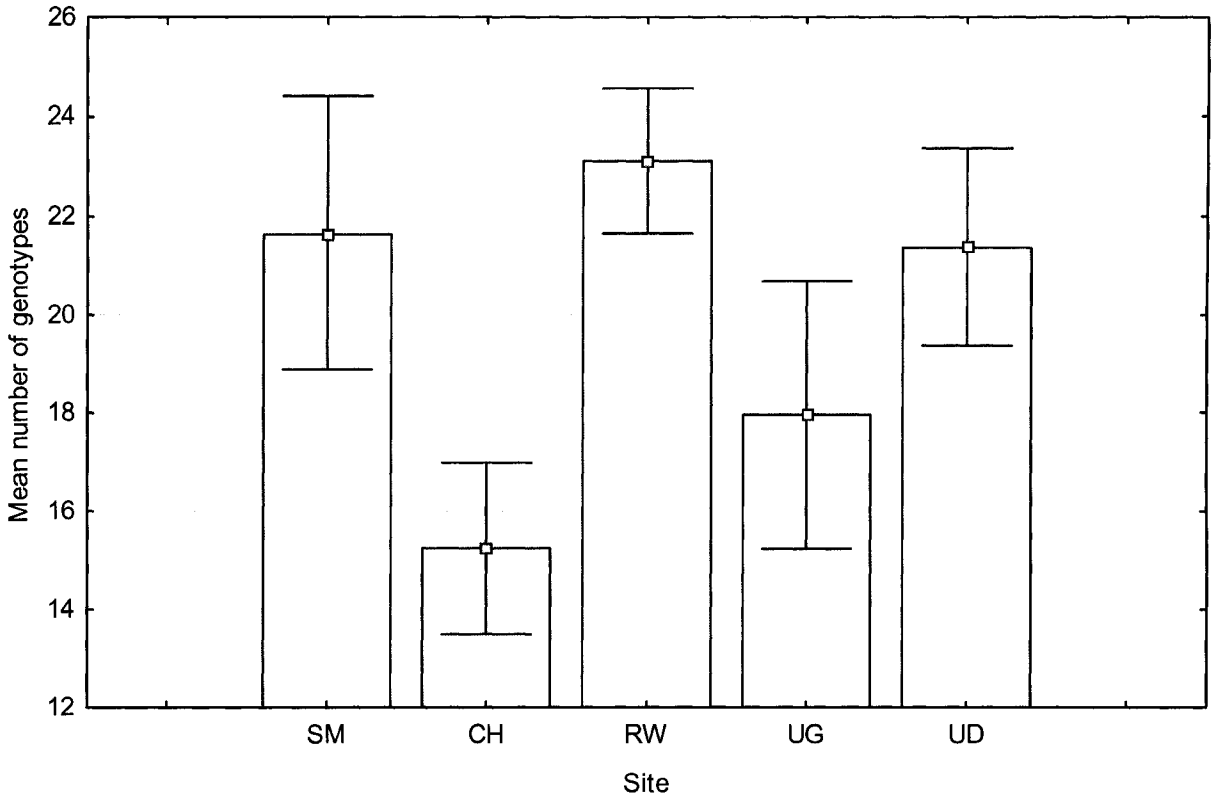

Fig 33: Mean number of *nif*H genotypes (TRFs) at all sites ($p<0.001$, confidence interval = 0.95).

Significant differences were detected in the number of *nif*H genotypes between upper and lower samples (Figure 34). Unexpectedly, there were more genotypes in lower soil samples at the CH and at both Upland sites. There was significantly greater genotype richness in surface soils at SM (27.3 vs. 16.3, $p<0.001$) and RW (25.1 vs.21.5, $p=0.012$).

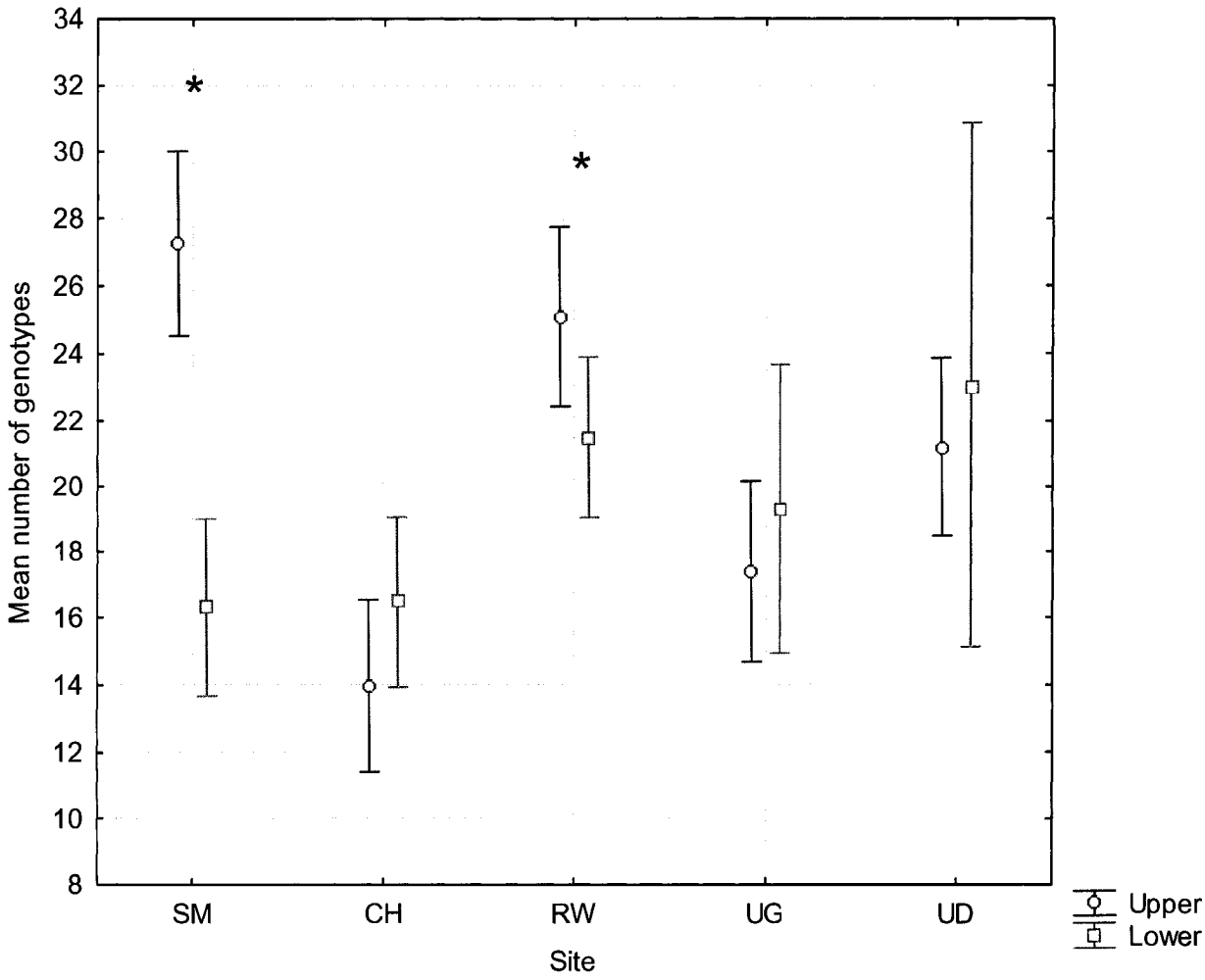

Fig 34: The mean number of *nif*H genotypes differed by depth over all sites (p<0.000001, CI=0.95). Significant differences at individual sites are marked with an asterisk (p<0.05).

Significant differences were detected in the number of *nif*H genotypes between control and OTC plots (Figure 35). With the exception of the UG site, there were fewer genotypes in OTC treatment samples. One-way ANOVA confirmed differences in genotype number at SM (25.4 vs. 17.4, p=0.0029), RW (25.3 vs. 20.7, p=0.0012) and UG (14 vs. 23.6, p=0.0006).

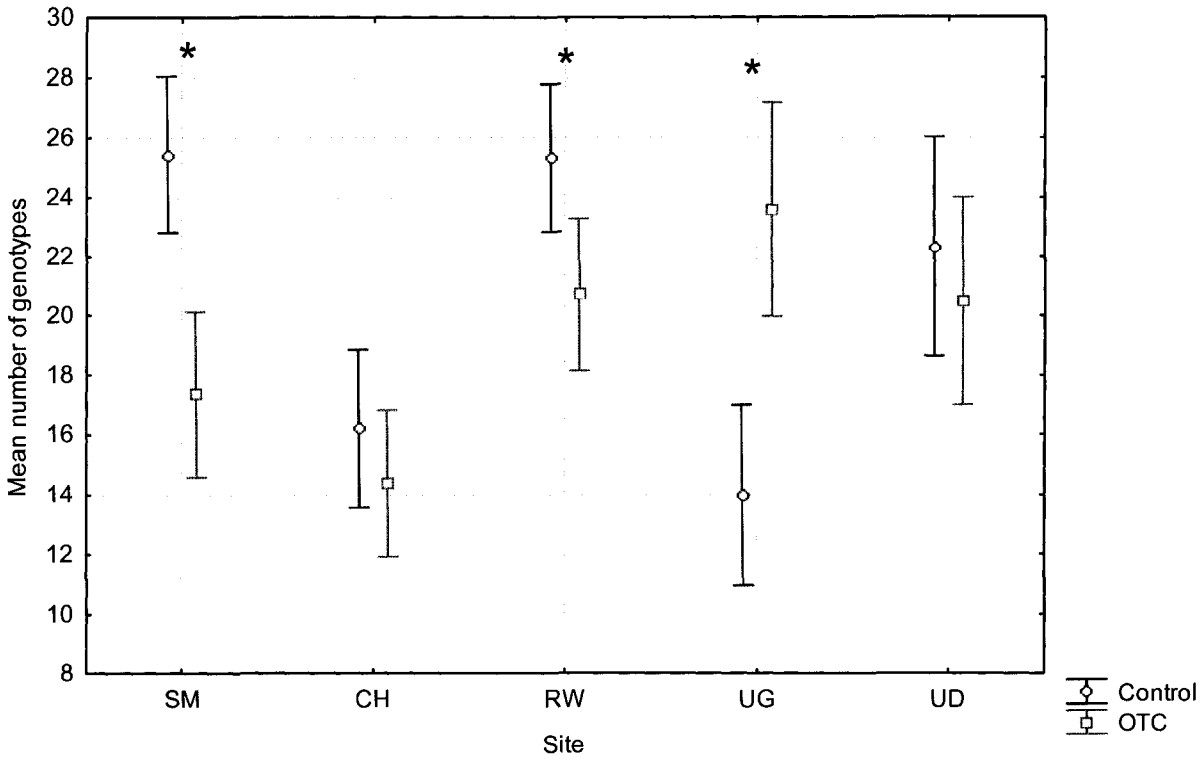

Fig 35: The mean number of *nif*H genotypes differed by treatment over all sites (p<0.000001,CI=0.95). Significant differences at individual sites are marked with an asterisk (p<0.005).

2.4. Discussion

2.4.1. Methodological considerations

We are assuming that each terminal restriction fragment (TRF) reflects a different bacterial genotype so that an increase or decrease in the frequency, abundance or overall number of these TRFs is a valid measure of genotypic frequency, abundance and/or richness (Tiedje *et al.* 1999). We also assume that uncut gene fragments TRF252 (*nos*Z) and TRF384 (*nif*H) mean that no restriction site exists in these genotypes. It is possible that more than one genotype corresponds to one restriction fragment, including the uncut fragment, so that genotypic diversity based upon the number of TRFs could be underestimated (Dunbar *et al.* 2000; Wolsing and Priemé 2004). However, there is no reason to believe that any underestimate would be biased according to site, depth or treatment, so even if genotypic diversity were underestimated, comparisons should remain valid.

Soil cores were retrieved from plots that had undergone experimentation for over a decade (Freedman *et al.* 1994). The destructive nature of soil sampling could result in disturbance that affects microbial communities. However, Deslippe *et al.* (2005) conducted disturbance experiments at Alexandra Fiord sites and confirmed that previous soil excavation had no effect on *nif*H community structure, so we would not expect this to be a factor at our study sites.

The Sedge Meadow soils had high organic matter content and DNA extractions required 1:100 dilution in order to overcome the interference of humics in PCR reactions of both functional genes. A humic acid purification step may have allowed amplification without dilution, but the process can result in decreased product yield (Brodie *et al.* 2002).The Upland soils were very dry with little organic matter and although consistent PCR results were obtained for *nos*Z, few or no *nif*H amplification products were retrieved from lower samples, even with multiple attempts. We assume that this was not a failure in our methods, but that the number *nif*H genes were often below detectable limits or simply not consistently present in samples from this depth at UD.

2.4.2. Did the denitrifying and nitrogen-fixing communities respond similarly or differently to experimental factors?

The overall differences between sites were similar for both *nos*Z and *nif*H, but were not as clearly defined for nitrogen fixers, especially when assessed by gene frequency. Where *nos*Z TRF frequency was distinct between sites due to one or a few dominant genotypes, *nif*H TRFs were shared across all sites with small changes in the frequency distribution. In contrast, differences in the relative abundance of shared *nif*H TRFs were more apparent between sites. Subtle changes in the frequency and abundance of *nif*H TRFs between sites were consistent with findings of uniform gene distribution and identical sequences despite large geographic distance found by other researchers (Poly *et al.* 2001, Rösch *et al.* 2002). Alternately, enormous variation in *nos*Z TRF distribution at distances of centimeters, meters and especially kilometers has been found in

marine studies suggesting that denitrifier genotypes are unique at each location (Scala and Kerkhof 2000). For both genes, the SM site stood apart from the other four, but *nos*Z genotype richness was very low where *nif*H richness was high. There were no significant differences between CH and RW (*nos*Z) or UG and UD (*nif*H). *nos*Z genotype richness was high at both CH and RW, but *nif*H richness was low at CH. Alternately, *nif*H genotype richness was intermediate at both UG and UD, but *nos*Z richness was very low at UG.

*nos*Z genotypic richness at each site seemed to affect the number of genotypes at other levels. For example, sites with low or moderate *nos*Z richness (SM, UG, and UD) showed significant or unexpected changes in the number of TRFs at two depths and between treatments. These homogeneous sites have unique ecological characteristics that only a few, dominant *nos*Z genotypes can exploit (anaerobic at SM, acidic at UG, and dry at UD). As a result of this, the community changed enormously over vertical microsites and with warming. Sites with high *nos*Z richness (CH, RW) showed little or no significant change at other levels. This suggests that sites with high *nos*Z richness and uniform vertical gene distribution are less likely to experience a change in community structure due to disturbance. This could be due to the fact that the denitrifier communities at CH and RW, due to the heterogeneous nature of these sites, already contain members that are able to exploit all reasonable niches on the lowland, including those that have changed due to OTC treatments.

*nif*H genotype richness at each site seemed to have some influence on number of genotypes at two depths or due to treatment, although the effects were not consistent with those described for *nos*Z. For example, sites with low or moderate *nif*H richness (CH and UG) showed either no significant changes or showed differences in richness that were not predicted. Sites with a high number of *nif*H genotypes (SM and RW) showed significant changes due to depth or treatment. This suggests that the majority of these genotypes were rare and able to exploit only a narrow environmental niche.

Both *nos*Z and *nif*H genotypes were affected overall by depth, particularly at the SM site where each community experienced a significant decline in genotype richness in lower samples. At SM, the frequency of *nif*H genotypes clearly shifted among common TRFs; changes in the most abundant *nif*H TRFs show that one distinct genotype structured upper or lower samples. The frequency of *nos*Z TRFs was not significantly affected at SM, but the relative abundance of TRFs also shifted dramatically to one dominant genotype in lower samples. There was a significant decrease in *nos*Z genotype richness in lower samples at the UD site, but this could not be confirmed for *nif*H as limited PCR amplification of lower samples caused enormous variation in the *nif*H data. Where there was a shift in the distribution of both frequent and abundant *nos*Z genotypes between upper and lower samples at the UD site, there was, in addition to a shift, a loss of *nif*H genotypes from lower samples at the UG site. Interestingly, there was a corresponding gain in the mean number of *nif*H genotypes in lower samples at

this site, plus a loss of genotype richness in lower samples from the RW site that was not detected in NMS ordinations.

The presence or absence of an alternative denitrification marker (*nir*S) has been used to investigate changes in community structure in ocean sediment cores based upon the transition to an anaerobic environment as depth increased (Braker *et al.* 2001). Only a slight decrease in denitrifier diversity was detected in deeper samples even though a strong redox gradient was present suggesting that the microbial community was not vertically structured. Enzyme analysis and gene probing of forest soils does suggest that denitrifier abundance decreases with depth even though the deepest samples (-25 cm) had very low levels of oxygen and should have selected for denitrifiers (Mergel *et al.* 2001, Rösch *et al.* 2002). The SM soil cores were uniformly anaerobic throughout, so any differences detected could have been due to the change from organic to mineral soils and the subsequent changes in soil chemistry and nutrient availability. The UD soils were uniformly mineral, but a shallow rhizosphere suggests a lack of nutrient availability beyond 8 cm from the surface.

Studies of N-poor acid forest soils have indicated a distinct difference in *nif*H RFLP patterns between litter layers and soil samples (Widmer *et al.* 1999), and a decrease in pattern complexity from shallow to deep soil samples (Shaffer *et al.* 2000). In some cases genes appeared to be more abundant in litter versus soil, although the gene marker was shared by both samples, in others the genotype

was simply not present in the soil sample. Gene probing of forest soils did suggest that *nif*H was more abundant in the top 5cm than in deeper samples (Mergel *et al.* 2001, Rösch *et al.* 2002).

There was a significant overall difference in both *nos*Z and *nif*H genotype richness between control plots and OTCs. In general, *nos*Z genotype richness decreased with warming treatments at all sites, but the change was only significant at SM, UG, and UD. The mean number of *nif*H TRFs also decreased significantly with treatment at SM and RW, and was lower, though not significantly, at CH and UD. We did not expect to see the significant increase in genotype richness with treatment at UG.

If the soils of Alexandra Fiord are N limited (Muc *et al.*, 1994), and OTCs effectively raise soil temperatures enough to promote organic matter decomposition and net N mineralization (Berendse and Jonasson 1992, Paul and Clark 1996, Nadelhoffer *et al.* 1992), the increase in nutrient availability would affect denitrifier and diazotroph communities differently based upon their physiological requirements. Direct increases in both denitrification and nitrogen fixation are possible due to higher enzymatic activity at increased temperatures (Chapin and Bledsoe 1992, Paul and Clark 1996). Additionally, anaerobic denitrifiers and aquatic nitrogen fixers should flourish in the wetter environment that is predicted but not accomplished with OTCs. Photosynthetic diazotrophs, such as the cyanobacteria that dominate arctic systems (Chapin and Bledsoe

1992, Liengen 1999, Paul and Clark 1996), should also benefit from increases in CO_2 production associated with OM turnover (Chapin and Bledsoe 1992).

It is possible that the same conditions that promote microbial growth in these changed systems may also inhibit the processes mediated by them. For example, net N mineralization is predicted, but N-fixation is inhibited by the presence of available N (Paul and Clark 1996). It is possible that the N-fixation process and diazotroph community structure are unrelated (Piceno and Lovell 2000a), but in theory an N rich habitat should result in low *nif*H diversity (Zehr *et al.* 2003). Alternately, NH_4^+ is required by nitrifying bacteria that oxidize it to NO_3^-, the substrate for denitrification (Paul and Clark 1996, Nadelhoffer *et al.* 1992). Any potential increases in this process will be moderated by the success of aerobic nitrifier communities in an environment predicted to be wetter (Nadelhoffer *et al.* 1992). If excess NO_3^- is the result, then anaerobic soil conditions greater than 5 °C, with a pH range of 6-8 should be ideal for denitrification to occur (Paul and Clark 1996). Additionally, changes in the proportional abundance of dominant TRFs have been detected between different levels of denitrification activity in two different vegetation types (Rich *et al.* 2003), although this finding was not repeatable (Rich and Myrold 2004).

Recent studies have not confirmed that net N mineralization will be affected by short term increases in soil temperature of a few degrees Celsius (Jonasson *et al.* 1999, Schmidt *et al.* 1999). Rolph (2003) could not show that an increase in

mineralization had occurred due to long term OTC treatment at the Alexandra Fiord site although data from the 2001 growing season indicate that soil temperatures were raised by less than one degree Celsius.

2.4.3. What experimental factors most influenced community structure?

Site and depth were more important than treatment in structuring both denitrifying and nitrogen-fixing microbial communities; of the first two factors, site was more important than depth.

Site

The sites vary in both biotic and abiotic properties, which results in homogeneous or heterogeneous environments with a range of substrate availability. These dissimilarities combine to create the unique nature of SM, the commonalities between CH and RW, and the divergent nature of the upland sites from those on the lowland and from each other. As discussed, the community structure of each functional gene responded differently to these changes reflecting the requirements of either denitrifier or nitrogen fixer groups.

The SM site is unique within the study area due to the hydric and often flooded conditions. The dominant sedge vegetation created a distinctive *nosZ* community in terms of gene frequency, abundance, and richness. Low *nosZ* genotype numbers at this site may be a function of homogeneous conditions that do not necessitate a diverse denitrifier community to exploit them (Callaghan *et al.*,

2004). Alternately, high C:N plus anaerobic conditions may not favour nitrification and thus limit the substrates required for denitrification (Nadelhoffer *et al.*1992). *nif*H genotypic richness was high at the SM site where diverse, aquatic cyanobacteria may be dominant (Callaghan *et al.* 2004, Chapin and Bledsoe 1992, Zehr *et al.* 1998) and where there was sufficient C plus N-limitations due to a large amount of poorly decomposed plant material (Klady, personal correspondence June 2006). It is not uncommon to find diverse *nif*H communities in anaerobic environments (Ueda *et al.* 1995, Zehr *et al.* 1998).

CH and RW shared many *nos*Z genotypes in common, and grouped together when gene frequency and abundance were examined. Though the RW has the highest plant diversity of all sites in the study area, *nos*Z richness was similar between CH and RW; comparable soil moisture regimes, common plant species, and similarly low soil pH could contribute to these parallels. It is possible that greater variation in the soil environment at these sites has required a greater variety of *nos*Z in order to exploit all opportunities for denitrification. The observations of Brodie *et al.* (2002) based upon bacterial communities in disturbed grassland, and Stres *et al.* (2004) based upon denitrifiers in forest soils were not mirrored at CH or RW as denitrifier richness was relatively high despite a mixed plant community and acidic soils. Low *nif*H genotype richness at CH could not be explained by soil moisture, organic matter content, or pH as the site has intermediate values of these factors between all three on the lowland. The hydric SM soils have an average pH of 6.25 while the mesic-xeric soils of RW

have an average pH of 4.9; CH is hydric-mesic with an average pH of 5.15. The RW site had the greatest number of *nif*H TRFs due possibly to the influence of high plant diversity. Variable soil conditions due to plant feedbacks at this site may also have promoted the development of a diverse *nif*H community.

The Upland sites are remarkably different from those on the lowland due to the dry nature of their soils and relatively sparse vegetation. They share a few plant species in common, but differ greatly in soil pH; the granitic site averages pH 5.2 and the dolomitic site is much more alkaline at pH 7.9. The acidic nature of the UG soils could have contributed to the lack of *nos*Z genotype richness when compared to the adjacent UD site, although low pH did not structure *nos*Z communities on the lowland. These observations suggest abiotic properties of the dry soil environment may have had more influence on the structure of upland *nos*Z communities. Soil pH may also explain lower *nif*H richness at UG when compared to adjacent UD as the plant communities, mineral soils, and moisture regimes are similar at these sites.

Past experiments have used frequency and abundance of the TRFs from other denitrification gene markers to explore diversity across spatial scales (Braker *et al.* 2000, 2001). They confirmed that denitrifier gene communities were unique to the environment from which they were sampled, with only a few genes shared between locations. Similarly, *nif*H TRFs that were found to be dominant at one

site were often less frequent, or less abundant, or not present at all at adjacent sites with different plant and soil characteristics (Shaffer *et al.* 2000).

Meadow soils with lower C:N and higher pH than adjacent forest plots have demonstrated distinct *nos*Z communities (Rich *et al.* 2003). Few TRFs were shared between the meadow and forest sites which differed in predominant vegetation as well as N availability. An increase in *nos*Z diversity in disturbed, amended soils was also related to higher pH than adjacent native plots (Stres *et al.* 2004). Brodie *et al.* (2002) found that the number of bacterial TRFs increased as the grassland community became more uniform and as both pH and N, P, and K increased. Other groups have noted that bacterial community diversity decreases when the environment is more homogeneous (Callaghan *et al.*, 2004). Higher pH and available N seem to be more consistent factors associated with high microbe diversity than site vegetation. Moreover, it appears that denitrifier communities are structured by not only the amount of N available, but also whether it is in organic or mineral form (Wolsing and Priemé 2004).

Acidic forest soils showed less abundance and diversity of denitrification genes than adjacent marsh soils with high organic matter content (Priemé *et al.* 2002). This contradicts the finding of low genotypic richness of *nos*Z in the thick organic and alkaline soils of the Sedge Meadow site, where the anaerobic conditions should be an advantage for denitrifiers. That this site stood out from all others in

terms of frequency and abundance of TRFs is consistent with literature that suggests *nos*Z is habitat specific (Rich *et al.* 2003, Rich and Myrold 2004, Rösch *et al.* 2002, Stres *et al.* 2004), but the adjacent lowland sites are drier and more acidic, yet show greater *nos*Z richness. On the lowland, it appears that pH and organic matter content are not strong drivers of high *nos*Z richness, but it is important to note that large seasonal shifts have been detected in denitrifying gene communities (Wolsing and Priemé 2004) and that this study examined samples taken only once during the summer growing season.

*nif*H gene communities are also known to vary over large and small spatial scales (Poly *et al.* 2001, Rösch *et al.* 2002) but the factors that control this variation are complex. Community structure is related to soil management and soil texture that influences inorganic N availability (Poly *et al.* 2001). *nif*H communities are habitat specific (Shaffer *et al.* 2000, Zehr *et al.* 1998) and most similar when their site characteristics are the same but neither plant cover nor soil chemistry could completely explain this (Poly *et al.* 2001).

Depth

Depth of the sample (i.e. upper versus lower section of the soil core) was also an important factor differentiating *nos*Z and *nif*H communities. Although both genes were affected, this separation was not detected at all sites, nor was it consistently found at treatment and OTC plots simultaneously.

*nos*Z relative abundance data showed dissimilarity between upper and lower samples at Sedge Meadow OTCs only. One SM *nos*Z TRF was most abundant at both depths but its relative contribution was much greater in lower samples. *nos*Z data for the Upland Dolomite site showed that the distribution of most frequent TRFs changed between depths at both control plots and OTCs but the most abundant *nos*Z TRFs were different only at control plots. Overall, upper samples appeared to have a more uniform distribution of *nos*Z TRFs, while lower samples were dominated by one (TRF 251).

*nif*H frequency and relative abundance also varied between depths at the SM site; differences were detected at both control plots and OTCs. Each depth was dominated by one particularly abundant genotype and had a unique distribution of less abundant TRFs. Upland Granite *nif*H frequency and relative abundance differed at control plots only; overall, the most frequent or abundant TRFs in upper samples were not represented at all in the lower soils.

In general, there was greater genotype richness in surface soils. Significant differences were detected in the number of *nos*Z genotypes between upper and lower samples at SM and UD and in the number of *nif*H genotypes between upper and lower samples at SM and RW.

The SM site had a large range in terms of depth between upper and lower soil samples (0-5 cm deep and 39-44 cm deep, respectively) but this did not differ

greatly from other lowland sites. More importantly the soil core itself was not as uniform as those from CH and RW; the upper section was wet organic peat, while the lower part of the core had a greater proportion of mineral soil. Differences in the relative abundance and genotypic richness of *nos*Z TRFs between upper and lower samples reflected the distinct habitats at either end of the SM soil core. UD cores, in contrast, were very shallow and uniform; the lower samples were only 8-13 cm from the surface. It is interesting that differences were found even though soil samples were in relatively close proximity. This site was the driest in the study area and it is possible that there was little interaction between surface and subsurface soil layers.

Differences in *nif*H gene frequency and abundance between upper and lower samples at the SM, plus differences in genotype richness at SM and RW may be partly explained by the vertical distance between sample locations as explained for *nos*Z. Additionally, a greater proportion of cyanobacteria would be expected in surface samples due to their phototrophic mode of nutrition (Paul and Clark 1996). The UG site had slightly more moisture than the adjacent dolomitic site, and the depth of soil cores averaged 30-35 cm. The distance between upper and lower samples in a relatively dry soil with a shallow root zone may have contributed to the differences in gene frequency and relative abundance detected. A unique community of organotrophic N fixers would be found adjacent to plant roots versus the soil beyond the influence of plant inputs (Paul and Clark 1996).

Treatment

The main objective of this study was to detect shifts in denitrifying and nitrogen fixing microbial communities after long term warming of arctic soils. Soil warming is a factor that can structure microbial communities. Increases in soil moisture and organic matter availability due to warmer temperatures in arctic systems may lead to higher rates of decomposition and nutrient cycling (Berendse and Jonasson 1992, Paul and Clark 1996, Nadelhoffer *et al.* 1992). It is important to note that increases in arctic air temperatures predicted by climate models and accomplished with warming experiments do not necessarily translate into proportional increases in soil temperatures (Jonasson 1993, Robinson 2002). At times, only the surface soils are affected by direct heating unless the site is very wet, and shading due to increased plant growth can prevent warming of the soil (Rolph 2003). This means that although enzymatic processes in soils may sometimes increase due directly to higher temperatures, and biogeochemical cycling may be stimulated by increases in substrate availability, there are indirect changes caused by warming that also contribute to changes in the soil environment.

Warming can cause shifts in plant, fungal and protozoan communities that further impact microbial communities. Favourable conditions that increase the abundance and activity of soil protozoa can result in increased grazing of soil microbes, directly affecting their number and distribution (Ruess *et al.* 1999). The

biomass of both saprotrophic and mycorrhizal fungi is positively affected by moderate increases in temperature and moisture, and changes in the community composition and function of fungal decomposers has been noted (Robinson 2002). Experiments have indicated that increases in fertilization and/or temperature can lead to greater plant productivity (Arft *et al.* 1999, Muc *et al.* 1994, Shaver *et al.* 2001) but loss of diversity (Chapin III *et al.* 1995). Passive warming experiments that raise air temperatures by 1 to 3°C resulted in decreased plant diversity and species evenness at a number of ITEX sites (Walker *et al.* 2006). Complex feedbacks to the soil environment due to shifts in plant and fungal community structure depend on changes in carbon allocation (Piceno and Lovell 2000b), species composition and litter quality (Shaver *et al.* 2001), and nutrient use efficiency (Berendse and Jonasson 1992), driven by long term changes in N mineralization and availability (Chapin III *et al.* 1995, Rolph 2003, Schmidt *et al.* 2002). Changes in N mineralization and availability were investigated after ten years of warming at the Alexandra site (Rolph 2003). Net N mineralization was not affected by warming treatments, but inorganic N availability was higher in OTCs throughout the growing season except at the upland dolomitic site and NH_4^+ increased with warming at the heath and willow sites (Rolph 2003).

Contrary to expectations, warming had the least effect of all factors on denitrifier and nitrogen fixer community structure. There was no separation of samples due to treatment detected at any site for either *nos*Z or *nif*H frequency or relative

abundance data. It is possible that NMS ordinations and PERMANOVA tests were not sensitive to changes in the frequency and/or abundance of rare genotypes. When genotypic richness was explored by comparing the number of TRFs between control plots and OTCs, a treatment effect was evident. Overall, it appears that rare species were lost and dominant species prevailed when disturbed by OTC treatments.

In general, there were fewer genotypes in treatment samples except for *nif*H TRFs at the UG site. The large, significant increase in the number of genotypes at UG OTCs is surprising since Rolph (2003) detected higher inorganic N at this upland site with warming suggesting that a diverse diazotroph community is not required. Alternately, the loss of genotype richness was significant for both genes at SM where Rolph (2003) found the greatest response to warming when nitrogen transformations were compared between control plots and OTCs. Specifically, both NO_3 and N immobilization increased at SM OTCs supplying more substrate for denitrification and suggesting an increase in microbial growth (Paul and Clark, 2006). It is possible that although warming at the SM created ideal denitrification conditions, only a few genotypes (from an already limited number) could exploit the altered environment. The nitrogen fixing community, although initially genotype rich at this site, experienced a large decrease in the number of genotypes suggesting an abundance of rare genotypes that are sensitive to disturbance. Significant TRF losses due to OTC disturbance were

also detected by changes in the number of *nos*Z genotypes at UG, and UD, and in the number of *nif*H genotypes at RW.

Long term disturbance of forest soils due to clearcutting leads to distinctly different *nif*H communities with new dominant TRFs and losses of previously dominant genotypes; additionally, these communities exhibit atypical seasonal variation (Shaffer *et al.* 2000). Based upon previous work, the authors suggested that a loss in diversity of *nif*H genotypes reduces the capacity for nitrogen fixation in disturbed systems. Deslippe (2004) also noted that *nif*H communities disturbed by warming experiments are more seasonally variable; we presume then, that although these communities are very adaptable, they are also less stable. Disturbance of marsh soils by long term fertilization leads to changes in *nif*H community composition and absence of genotypes due possibly to the loss of competitive advantage over other N-limited microbes (Piceno and Lovell 2000a). This artificial change in nutrient availability mirrors the changes predicted by long term soil warming and may explain why we observed a loss of genotype richness with OTC treatments.

Deslippe *et al.* (2005) suggested that *nif*H communities were structured by warming late in the growing season, and NMS ordinations showed differences between control plots and OTCs at lowland sites. Although samples for this investigation were collected at approximately the same time of year (July 18-27) at adjacent plots in the same study area, we did not detect a similar shift in *nif*H

gene frequency using the same statistical methods. This suggests that there may be seasonal shifts in these communities that are detectable with multiple sampling times over the growing period, but that the changes do not persist from year to year. In this case, short term changes due to dynamic soil processes such as N transformations may be more of a factor than long term community shifts due to persistent changes in soil chemistry from altered plant inputs (Shaver, 2000). This has not been found experimentally for *nif*H; the seasonal community structure appears to be very stable despite repeated sampling over weeks to months (Poly *et al.* 2001, Shaffer *et al.* 2000, Widmer *et al.* 1999), even with increased nitrogen (Piceno and Lovell 2000a) or reduced carbon availability (Piceno and Lovell, 2000b).

In contrast, enormous seasonal variation has been detected in *nos*Z gene markers from ocean sediment samples measured over the course of one year (Scala and Kerkhof 2000) and studies of soil thawing effects on denitrifying communities have demonstrated that short term community shifts can also be detected by measuring gene expression with microbial RNA (Sharma *et al.* 2006). Sharma and colleagues (2006) found that the diversity of an alternate denitrification gene marker (*nir*S) appeared to increase as thawing progressed and microbial activity was stimulated; although *nos*Z DNA was also present, no *nos*Z transcripts were detected as N_2O accumulated. It has been suggested that seasonal and/or geographic temperature variation can structure nitrifier communities either directly or by affecting N availability (Avrahami *et al.* 2003,

Avrahami and Conrad 2003). It would seem that the successful detection of seasonal variation in nitrogen cycling gene community structure (due directly or indirectly to temperature manipulations) depends on the timing and frequency of sampling, plus the molecular technique and gene marker employed.

Net N mineralization is greater in wet sedge-dominated tundras than in other arctic ecosystems and has been shown to increase with warming, subsequently doubling the inorganic N pool and resulting in an excess of mineral N but this increase appeared to benefit the plant community more than it did the microbes (Schmidt *et al.* 2002). This explains why even though the microbial community experienced a strong disturbance effect at SM, gene richness was not affected positively by the increased nutrient availability associated with OTC treatments. Other subarctic studies have shown no significant increases in net N mineralization with experimental soil warming of 1-2°C (Jonasson *et al.* 1993) and only moderate average increases with soil temperature changes of 0.3-5.1°C (Rustad *et al.* 2001). Arctic mineralization rates could more than double if soil temperatures reach or exceed 10°C especially where tundra soils have high organic matter quality (Nadelhoffer *et al.* 1991). Interestingly, no significant changes in gene richness were detected at CH where average soil temperatures measured in 2001 at Alexandra Fiord (Rolph 2003) were increased by only 0.2°C with OTC treatment. TRF loss was noted only for *nif*H at RW where the same data shows an average increase of 0.5°C and genotype richness decreased for both genes at SM where the greatest temperature increase was recorded

(0.7°C). Hollister *et al.* (2006) also found that OTC effect was variable at different tundra sites, and differed greatly from day to day and from year to year. Average soil temperatures at -10 cm in July ranged slightly from 0.3°C to 0.6°C at dry heath sites, and enormously from -0.8°C to 0.7°C at wet sedge sites.

Increases in net mineralization and the subsequent availability of NH_4 are highly dependent upon rates of microbial immobilization (Jonasson *et al.* 1993, Rustad *et al.* 2001), and will affect soil nitrogen cycles such as nitrification, denitrification, and nitrogen fixation. Nitrogen fixation should be inhibited by the presence of its products NH_4^+ and NO_3^- (Paul and Clark 1996), but rates were not affected by the presence of nitrate in field samples of high arctic cyanobacteria (Liengen 1999). It is known that nitrification and denitrification are stimulated by the availability of these substrates (Nadelhoffer *et al.* 1992, Nicolaisen *et al.* 2004), and that the amount of NH_4-N can be far greater than NO_3-N during the growing season in arctic soils (Schmidt *et al.* 2002). Although enzymatic processes are accelerated in general by increases in temperature, it is possible that a warmer soil environment will become more favourable for denitrification but select against nitrogen fixers (Paul and Clark 1996).

2.4.4. What measures of community structure are most affected by environmental factors?

We measured diversity in three ways: genotype richness, genotype frequency and genotype relative abundance. Although genotype richness was the coarsest measure, it was the most sensitive to rare genotypes, and the only one that was able to distinguish a treatment effect.

Relative abundance measures are confounded by the presence of rare species because they create noise in the data, and frequency measures are biased towards evenly dispersed sample units (McCune and Mefford 1999, McCune and Grace 2002). We propose that NMS ordinations and PERMANOVA tests did not detect changes in rare genotypes or clustered individuals because, in these analyses, the data are equally weighted and more strongly influenced by dominant TRFs (McCune and Grace 2002, Anderson 2005). By measuring the differences in absolute number of TRFs (richness) we saw how the contribution of all genotypes was affected by site, depth, and treatment (Dunbar *et al.* 2000).

Genotype richness is strongly affected by the environmental factors that make each site unique. The mean number of *nos*Z TRFs ranged from only 5.8 at the Upland Granite site to 18.8 at the Cassiope Heath site while the mean number of *nif*H TRFs also varied between sites, from 15.2 at Cassiope Heath to 23.1 at Riverside Willow. Depth also affected both *nos*Z and *nif*H richness. Significantly

fewer genotypes were found in lower samples at SM (both genes), UD (*nos*Z), and RW (*nif*H). A significant warming effect on gene richness was detected at three of five sites for both microbial communities. The number of *nos*Z genotypes decreased with treatment at SM, UG, and UD while *nif*H richness declined at SM and RW, but increased at UG.

Genotype frequency was not as sensitive as relative abundance to differences between sites, but it did give us more information than genotype richness about the presence or absence of individual TRFs. For example, although the number of different genotypes may have been low in each sample from one site (resulting in low mean number of genotypes for that site), the frequency data showed us that when pooled, many more TRFs were actually represented. Because the most frequent TRFs (ie. the top 20) were often at least present across all sites, the differences between sites is due to small changes in the frequency distribution of common genotypes. The relative abundance data shows more clearly how each site is structured by one or a few dominant TRFs. The dominant TRF(s) plus a unique distribution of other genotypes in very low abundance create(s) a distinct community at each site.

Genotype richness, frequency and relative abundance were all sensitive to sample depth, but not at all sites, and not to the same degree. There was a significant decrease in the number of genotypes with increasing depth at two of

five sites for each gene. Interestingly, a loss of approximately two *nos*Z genotypes at SM was not detected as a change in frequency, while a loss of approximately four *nos*Z genotypes at UD was documented in frequency ordinations. All of the top 20 most frequent UD *nos*Z TRFs were represented in upper and lower samples, but their distribution was altered. A significant loss of *nif*H genotype richness was detected at SM and RW, but the change was fewer than four TRFs at the latter site and was not reflected in the frequency data. Instead, the frequency distribution of the top 20 SM and UG *nif*H TRFs was shown to have shifted between upper and lower samples. Overall, *nif*H TRF frequency was uniform across upper samples and variable in lower samples; at the UG site, some dominant TRFs were no longer present with increased depth.

Depth affected *nos*Z relative abundance data at both the SM and UD site. Unlike the frequency data, the distribution of TRFs did not simply shift among the top 20. Upper samples were initially more uniform, but there remained only one or a few dominant TRFs in the deeper samples while the rest were lost. The relative abundance of *nif*H TRFs was structured by depth at both the SM and UG sites. At SM, none of the top 20 TRFs were lost, but two very different genotypes were dominant in upper versus lower samples. Two different pairs of TRFs dominated either upper or lower samples from UD.

Chapter 3. Summary

3.1. Conclusions

The objective of this study was to detect shifts in denitrifying and nitrogen fixing soil microbial communities by measuring changes in functional gene frequency, abundance and/or genotypic richness using the genetic markers *nosZ* and *nifH* respectively. The study area encompassed five high arctic sites that differed by dominant plant community, parent material and/or soil moisture and that had been subjected to a long term warming experiment. We investigated differences in these gene communities due to site, depth, and treatment (warming).

The denitrifying and nitrogen-fixing communities respond both similarly and differently to experimental factors

The overall differences between sites were similar for both *nosZ* and *nifH*, but were not as clearly defined for nitrogen fixers. *nosZ* communities were more distinct between sites due to one or a few dominant genotypes while *nifH* TRFs were shared across all sites with subtle changes in their distribution. For both genes the SM site was unique, but *nosZ* genotype richness was very low where *nifH* richness was high. Both communities experienced a significant decline in genotype richness in lower samples and due to warming treatments at this site. Sites with low or moderate *nosZ* richness showed significant or unexpected changes in the number of TRFs at two depths and between treatments; alternately, sites with a high number of *nifH* genotypes showed significant changes due to depth or treatment.

Site most influenced community structure

The community structure of *nosZ* and *nifH* functional genes responded differently to the biotic and abiotic properties of each site reflecting the requirements of either denitrifier or nitrogen fixer groups respectively. This was most apparent at the SM site where homogeneous anaerobic conditions created distinct TRF communities. Denitrifier diversity may have been limited by the absence of a favourable nitrifier environment and subsequent lack of substrate for denitrification. Diverse diazotrophic cyanobacteria appear to have benefited from the aquatic, N-poor habitat.

The unique nature of the *nosZ* communities at each site confirmed that the distribution of this gene is habitat specific (Rich *et al.*, 2003; Rich and Myrold, 2004; Rösch *et al.*, 2002; Scala and Kerkhof, 2000; Stres *et al.*, 2004). Overall, *nosZ* richness was lowest where ecological conditions were most extreme and relatively high where conditions were moderate.

That each site shared common genotypes in varying proportions confirms that dominant *nifH* TRFs at one site may be absent, less frequent or less abundant at an adjacent site when predominant vegetation and soil properties differ (Poly *et al.*, 2001; Rösch *et al.*, 2002; Shaffer *et al.*, 2000). Alexandra sites with the greatest *nifH* richness were those with highly selective conditions.

Genotype richness was sensitive to all environmental factors

Genotype richness was affected by the environmental factors that made each site and depth unique. For both functional genes, the number of TRFs differed between sites and decreased overall in lower samples. When genotypic richness was explored by comparing the number of TRFs between control plots and OTCs, it appeared that rare species were lost and dominant species prevailed when disturbed by warming treatments.

3.2. Microbial community structure vs. physiological function

Callaghan *et al.* (2004) suggest that arctic environmental conditions restrict the metabolic potential of very diverse arctic microbial communities; enzymatic activity would easily approach that of boreal forests with forecasted increases in temperature. Unfortunately, the link between rates of microbially mediated processes and changes in microbial community structure (measured in terms of genetic diversity) are not well established. Changes in physiological function have been noted without affecting diversity at the genetic level (Gomez *et al.* 2004, Nicolaisen *et al.* 2004), and community shifts that do not affect microbial activity have been documented (Avrahami and Conrad 2003, Deslippe 2004). It has been suggested that biotic and abiotic environmental factors can structure the genetic composition of a system, but that gene selection is not the only driver of microbial diversity (Zehr *et al.* 2003).

3.3. Future research

This study would have benefited from a number of additional and parallel experiments that should be incorporated into future research. Plate counts to determine number of culturable bacteria, substrate-induced respiration for total microbial biomass and Biolog assessment of community profiles would be useful additions if only to help characterize the arctic microbial community. Physiological activity could be measured with enzyme analyses or other assays for total microbial activity (ie. denitrifying enzyme assay (DEA) for denitrification potential, gas chromatography (GC) for N_2O release, and acetylene reduction assay (ARA) for rate of N fixation). Phylogenetic information via culturing, cloning and sequencing would also be a fundamental addition to the study, and real-time PCR for gene expression would be an ideal tool if the objective is to truly link genetic diversity with physiological function.

Soil chemical analyses from the 2004 sampling season (pH, C:N, OM, NH_4, NO_3, P, K) will be invaluable additions as overlays to NMS ordinations in order to more thoroughly compare sites and elucidate factors that drive the differences that we detected.

We have learned that both *nosZ* and *nifH* communities are strongly structured by the environmental conditions of the site from which they are sampled. Although it is not clear exactly what biotic or abiotic factors drive these patterns, it is worthwhile examining study sites that are very similar to one another if the

objective is to measure only the effects of warming treatments. The magnitude of OTC disturbance is unique to each habitat and significant temperature changes below the soil surface must be confirmed. Additionally the method of measurement in important to determine relevant changed to microbial communities. For example, a change in the relative abundance of a particular genotype may indicate a community shift but not necessarily a change in the physiological process it is associated with. Alternately, mean loss of genotypes, although a coarse measure, may give more information about the resilience of a community to disturbance and the potential for microbial activity.

Literature Cited

Arft AM, Walker MD, Gurevitch J, Alatalo JM, Bret-Harte MS, Dale M, Diemer M, Gugerli F, Henry GHR, Jones MH, Hollister RD, Jónsdóttir IS, Laine K, Lévesque E, Marion GM, Molau U, Mølgaard P, Nordenhäll U, Raszhivin V, Robinson CH, Starr G, Stenström A, Stenström M, Totland Ø, Turner PL, Walker LJ, Webber PJ, Welker JM, Wookey PA. 1999. Responses of tundra plants to experimental warming: Meta-Analysis of the International Tundra Experiment. Ecological Monographs. 69: 491-511.

Anderson MJ. 2005. PERMANOVA: Permutational multivariate analysis of variance. University of Auckland, Department of Statistics.

Avrahami S, Conrad R, and Braker G. 2002. Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. Applied and Environmental Microbiology. 68: 5685-5692.

Avrahami S, Liesack W, Conrad R. 2003. Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. Environmental Microbiology. 5: 691-705.

Avrahami S, Conrad R. 2003. Patterns of community change among ammonium oxidizers in meadow soils upon long-term incubation at different temperatures. Applied and Environmental Microbiology. 69: 6152-6164.

Berendse F, Jonasson S. 1992. Nutrient use and nutrient cycling in northern ecosystems. In Arctic Ecosystems in a Changing Climate. Chapin III, Jefferies, Reynolds, Shaver and Svoboda, Eds. Academic Press, Toronto. 337-356.

Braker G, Zhou J, Wu L, Devol AH, Tiedje JM. 2000. Nitrite reductase genes (*nir*K and *nir*S) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. Applied and Environmental Microbiology. 66: 2096-2104.

Braker G, Ayala-Del-Río HL, Devol AH, Fesefeldt A, Tiedje JM. 2001. Community structure of denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by Terminal Restriction Fragment Length Polymorphism analysis of amplified nitrite reductase (*nir*S) and 16S rRNA genes. Applied and Environmental Microbiology. 67: 1893-1901.

Brodie E, Edwards S, Clipson N. 2002. Bacterial community dynamics across a floristic gradient in a temperate upland grassland ecosystem. Microbial Ecology. 44:260-270.

Callaghan TV, Björn LO, Chernov Y, Chapin T, Christensen TR, Huntley B, Ims RA, Johansson M, Jolly D, Jonasson S, Matveyeva N, Panikov N, Oechel W, Shaver G, Elster J, Henttonen H, Laine K, Taulavuori K, Taulavuori, E, Zöckler C. 2004. Climate change and UV-B impacts on arctic tundra and polar desert ecosystems: biodiversity, distributions and adaptations of arctic species in the context of environmental change. Ambio 33: 404-417.

Chapin DM, Bledsoe CS. 1992. Nitrogen fixation in arctic plant communities. In Arctic Ecosystems in a Changing Climate. Chapin III, Jefferies, Reynolds, Shaver and Svoboda, Eds. Academic Press, Toronto. 301-319.

Chapin III FS, Jefferies RL, Reynolds JF, Shaver GR, Svoboda J. 1992. Arctic plant physiological ecology in an ecosystem context. In Arctic Ecosystems in a Changing Climate. Chapin III, Jefferies, Reynolds, Shaver and Svoboda, Eds. Academic Press, Toronto. 441-451.

Chapin III FS, Shaver GR, Giblin AE, Nadelhoffer KJ, Laundre JA. 1995. Responses of arctic tundra to experimental and observed changes in climate. Ecology. 76: 694-711.

Deslippe J. 2004. Will climate change alter arctic nitrogen budgets? Impacts of warming and fertilization on nitrogen fixing microbial communities at Alexandra Fiord, Ellesmere Island, Nunavut. MSc Thesis. UNBC.

Deslippe JR, Egger KN, Henry GHR. 2005. Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic. FEMS Microbiology Ecology. 53: 41-50.

Dunbar J, Ticknor LO, Kuske CR. 2000. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Applied and Environ. Microbiol. 66: 2943-50.

Engebretson JJ, Moyer CL. 2003. Fidelity of select restriction endonucleases in determining microbial diversity by Terminal-Restriction Fragment Length Polymorphism. Applied and Environmental Microbiology. 69: 4823-4829.

Freedman B, Svoboda J, Henry GHR. 1994. Alexandra Fiord – An ecological oasis in the polar desert. In Ecology of a Polar Oasis. Svoboda and Freedman, Eds. Captus University Publications, London. 1-9.

Gomez EV, Garland JL, Roberts MS. 2004. Microbial structural diversity estimated by dilution-extinction of phenotypic traits and T-RFLP analysis along a land-use intensification gradient. FEMS Microbiol. Ecol. 49: 253-259.

Hassol SJ. 2004. ACIA, Impacts of a warming arctic: Arctic Climate Impact Assessment. Cambridge University Press.

Hollister RD, Webber PJ, Nelson FE, Tweedie CE. 2006. Soil thaw and temperature response to air warming varies by plant community: results from an open-top chamber experiment in northern Alaska. Arctic, Antarctic and Alpine Research. 38: 206-215.

Jonasson S, Havström M, Jensen M, Callaghan TV. 1993. In site mineralization of nitrogen and phosphorus of arctic soils after perturbations simulating climate change. Oecologia 95: 179-186.

Jonasson S, Michelsen A, Schmidt IK. 1999. Coupling of nutrient cycling and carbon dynamics in the Arctic, integration of soil microbial and plant processes. Applied Soil Ecology. 11: 135-146.

Kane DL, Hinzman LD, Woo M, Everett KR. 1992. Arctic hydrology and climate change. In Arctic Ecosystems in a Changing Climate. Chapin III, Jefferies, Reynolds, Shaver, and Svoboda, Eds. Academic Press, Toronto. 35-57.

Klady R. 2006. MSc Candidate. Geography Dept. UBC: Supervisor GHR Henry.

Labine C. 1994. Meteorology and climatology of the Alexandra Fiord lowland. In Ecology of a Polar Oasis. Svoboda, Freedman, Eds. Captus University Publications, London. 23-40.

Liengen T. 1999. Environmental factors influencing the nitrogen fixation activity of free-living terrestrial cyanobacteria from a high arctic area, Spitsbergen. Canadian Journal of Microbiology. 45: 573-581.

Maxwell B. 1992. Arctic climate: potential for change under global warming. In Arctic Ecosystems in a Changing Climate. Chapin III, Jefferies, Reynolds, Shaver, and Svoboda, Eds. Academic Press, Toronto. 11-34

McCune B, Grace JB. 2002. Analysis of ecological communities. MjM Software Design, Gleneden Beach, Oregon, USA.

McCune B, Mefford MJ. 1999. PC-ORD. Mulitivariate analysis of ecological data, Version 4. MjM Software Design, Gleneden Beach, Oregon, USA.

Mergel A, Schmitz O, Mallman T, Bothe H. 2001. Relative abundance of denitrifying and di-nitrogen fixing bacteria in layers of a forest soil. FEMS Microbiology Ecology. 36: 33-42.

Muc M, Svoboda J, Freedman B. 1989. Vascular plant communities of a polar oasis at Alexandra Fiord, Ellesmere Island. Can. J. Botany. 67: 1126-1136.

Muc M, Svoboda J, Freedman B. 1994. Soils of an extensively vegetated polar desert oasis, Alexandra Fiord, Ellesmere Island. In Ecology of a Polar Oasis. Svoboda, Freedman, Eds. Captus University Publications, London. 41-50.

Nadelhoffer KJ, Giblin AE, Shaver GR, Laundre JA. 1991. Effects of temperature and substrate quality on element mineralization in six arctic soils. Ecology. 72: 242-253.

Nadelhoffer KJ, Giblin AE, Shaver GR, Linkins AE. 1992. Microbial processes and plant nutrient availability in arctic soils. In Arctic Ecosystems in a Changing Climate. Chapin III, Jefferies, Reynolds, Shaver and Svoboda, Eds. Academic Press, Toronto. 281-300.

Nicolaisen MH, Risgaard-Petersen N, Revsbech NP, Reichardt W, Ramsing NB. 2004. Nitrification-denitrification dynamics and community structure of ammonia oxidizing bacteria in a high yield irrigated Philippine rice field. FEMS Microbiology Ecology. 49: 359-369.

Paul EA, Clark FE. 1996. Soil Microbiology and Biochemistry. 2nd Ed. Academic Press. Toronto.

Piceno YM and Lovell CR. 2000a. Stability in natural bacterial communities. I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. Microbial Ecology. 39: 32-40.

Piceno YM and Lovell CR. 2000b. Stability in natural bacterial communities. II. Plant resource allocation effects on rhizosphere diazotroph assemblage composition. Microbial Ecology. 39: 41-48.

Poly F, Ranjard L, Nazaret S, Gourbière F, Jocteur Monrozier L. 2001. Comparison of *nif*H gene pools in soils and soil microenvironments with contrasting properties. Applied and Environmental Microbiol. 67: 2255-2262.

Prescott LM, Harley JP, Klein DA. 1999. Microbiology. 4th Ed. McGraw-Hill. Toronto. Ch 22.

Priemé A, Braker G, Tiedje JM. 2002. Diversity of nitrite reductase (*nir*K and *nir*S) gene fragments in forested upland and wetland soils. Applied and Environmental Microbiology. 68: 1893-1900.

Rich JJ, Heichen RS, Bottomly PJ, Cromack Jr. K, Myrold DD. 2003. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Applied and Environmental Microbiology. 69: 5974-5982.

Rich JJ, Myrold DD. 2004. Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in

Oregon, USA. Soil Biology and Biochemistry. 36: 1431-1441.Robinson CH. 2002. Controls on decomposition and soil nitrogen availability at high latitudes. Plant and Soil. 242: 65-81.

Rolph SG. 2003. Effects of a ten-year climate warming experiment on nitrogen cycling in the high arctic tundra. MSc Thesis, University of British Columbia.

Rösch C, Mergel A, Bothe H. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Applied and Environmental Microbiology. 68: 3818-3829.

Ruess L, Michelsen A, Schmidt IK, Jonasson S. 1999. Simulated climate change affecting microorganisms, nematode density and biodiversity in subarctic soils. Plant and Soil. 212: 63-73.

Rustad LE, Campbell JL, Marion GM, Norby RJ, Mitchell MJ, Hartler AE, Cornelissen JHC, Gurevitch J. 2001. A meta-analysis of the response of soil respiration, net nitrogen mineralization and aboveground plant growth to experimental ecosystem warming. Oecologia 126: 543-562.

Scala DJ, Kerkhof LJ. 2000. Horizontal heterogeneity of denitrifying bacterial communities in marine sediments by Terminal Restriction Fragment Length Polymorphism analysis. Applied and Environmental Microbiology 66: 1980-1986.

Schmidt IK, Jonasson S, Michelsen A. 1999. Mineralization and microbial immobilization of N and P in arctic soils in relation to season, temperature and nutrient amendment. Applied Soil Ecology. 11: 147-160.

Schmidt IK, Jonasson S, Shaver GR, Michelsen A, Nordin A. 2002. Mineralization and distribution of nutrients in plants and microbes in four arctic ecosystems: responses to warming. Plant and Soil. 242:93-106.

Shaffer BT, Widmer F, Porteous LA, Seidler RJ. 2000. Temporal and spatial distribution of the *nif*H gene of N2-fixing bacteria in forests and clearcuts in western Oregon. Microbial Ecology 39:12-21.

Sharma S, Szele Z, Schilling R, Munch JC, Schloter M. 2006. Influence of freeze-thaw stress on the structure and function of microbial communities and denitrifying populations in soil. Applied and Environmental Microbiology 72: 2148-2154.

Shaver GR, Canadell J, Chapin III FS, Gurevitch J, Harte J, Henry G, Ineson P, Jonasson S, Melillo J, Pitelka L, Rustad L. 2000. Global warming and terrestrial ecosystems: a conceptual framework for anlysis. Bioscience 50: 871-882.

Shaver GR, Bret-Harte MS, Jones MH, Johnstone J, Gough L, Laundre J, Chapin III FS, 2001. Species composition Interacts with fertilizer to control long-term change in tundra productivity. Ecology. 82: 3163-3181.

Sterenberg VZ, Stone WE. 1994. Geology of the Alexandra Fiord lowland area Ellesmere Island, N.W.T. In Ecology of a Polar Oasis. Svoboda and Freedman, Eds. Captus University Publications, London. 18, 19.

Stres B, Mahne I, Avguštin G, Tiedje JM. 2004. Nitrous oxide reductase (*nosZ*) gene fragments differ between native and cultivated michigan soils. Applied and Environmental Microbiology. 70: 301-309.

Tiedje JM, Asuming-Brempong S, Nüsslein K, Marsh TL, Flynn SJ. 1999. Opening the black box of soil microbial diversity. Appl. Soil Ecol. 13: 109-122.

Throbäck IN, Enwall K, Jarvis A, Hallin S. 2004. Reassessing PCR primers targeting *nirS*, *nirK* AND *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology. 49: 401-417.

Ueda T, Suga Y, Yahiro N, Matsuguchi T. 1995. Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nif*H gene sequences. Journal of Bacteriology. 177: 1414-1417.

Walker MD, Wahren CH, Hollister RD, Henry GHR, Ahlquist LE, Alatalo JM, Bret-Harte MS, Clef MP, Callaghan TV, Carroll AB, Epstein HE, Jónsdóttir IS, Klein JA, Magnússon B, Molau U, Oberbauer SF, Rewa SP, Robinson CH, Shaver GR, Suding KN, Thompson CC, Tolvanen A, Totland Ø, Turner PI, Tweedie CE, Webber PJ, Wookey PA. 2006. Plant community responses to experimental warming across the tundra biome. PNAS. 103:5 1342-1336.

Widmer F, Shaffer BT, Porteous LA, Seidler RJ. 1999. Analysis of *nif*H gene pool complexity in soil and litter at a Douglas Fir forest site in the oregon cascade mountain range. Applied and Environmental Microbiology 65: 374-380.

Wolsing M, Priemé A. 2004. Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of *nir* gene fragments. FEMS Microbiology Ecology. 48: 261-271.

Zehr JP, Mellon MT, Zani S. 1998. New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nif*H) genes. Applied and Environmental Microbiology. 64:3444-3450.

Zehr JP, Jenkins BD, Short SM, Steward GF. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environmental Microbiology 5: 539-554.